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# PHENOL RED TRANSPORT IN THE PRONEPHROS AND MESONEPHROS OF THE DEVELOPING FROG (RANA PIPIENS)[1]

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TWO FIGURES

The cellular transport of phenol red has been demonstrated in mesonephric and metanephric kidneys, and the metabolic processes involved in the transport mechanism analyzed. The present report deals with phenol red transport in the pronephros.

Since the pronephros is the initial kidney in the renal developmental sequence, it is of interest to learn of the role it plays in the development of renal function. Both pronephros and mesonephros are present simultaneously for a considerable portion of the larval life of the frog. This has been shown in the analysis of the developmental morphology of the pronephros upon which the present experimental series is based. Since Gersh ('37) has shown that the mesonephros and metanephros of the chick and some mammals function synchronously, the pronephros and mesonephros of the larval anuran were examined to determine whether the same situation obtained.

Armstrong ('32) has demonstrated phenol red elimination in the pronephric tubules of *Fundulus heteroclitus,* and

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Swingle ('19) and Howland ('21) have shown that the amphibian pronephros becomes functional at an early stage of development. In the present study an effort has been made to correlate the onset of function, with regard to phenol red transport, with morphological development and cellular differentiation in the anuran pronephros.

Forster and Taggart ('50) have examined the metabolic processes involved in the cellular transport mechanism in the isolated tubules of the mesonephros of the flounder. A similar analysis has been carried out with tissue slices of the mammalian metanephros by Beyer, Painter and Wiebelhouse ('50). In the present investigations the isolated pronephros of the developing frog (*Rana pipiens*) has been utilized.

The literature concerning in vitro cellular transport studies has been reviewed by Forster and Taggart, and by Beyer, Painter and Wiebelhouse.

An analysis of phenol red transport in the larval mesonephros is included in the present report. This analysis was also undertaken to provide a direct comparison of the cellular transport mechanism of the mesonephros with that of the pronephros.

## METHODS

The procedures introduced by Forster ('48) and described in detail by Forster and Taggart ('50) were followed in the present studies. The renal organs were kept intact, however, as in the investigations of Richards and Barnwell ('27) in the adult frog kidney. Accurate observations of the extent of dye uptake by the individual tubules of the pronephros and mesonephros were possible because of the small size and simple tubular structures of these larval organs.

The solution developed by Forster ('48) was judged isotonic to the larval tissues since the explanted hearts and lymph hearts continued to beat for several hours in this medium. The renal organs were excised and placed in syracuse dishes containing the phenol red solutions. A minimum of two pronephroi and two mesonephroi were placed in each dish. Five dishes were utilized in the experiments; controls were placed in a dish containing the standard solution and the experimental agents were added to the phenol red solutions in the remaining dishes. Observations were made by placing a kidney in a thin layer of solution on a slide and inspecting the tubules at 30 × and 100 × magnifications. Under these conditions the phenol red in the solution was scarcely perceptible and the extent of dye accumulation by the individual tubules was observed clearly. Dye uptake was rated as maximal (++++) to minimal (+), as described by Forster and Taggart ('50). Maximal dye uptake was seen as an intense deep red color in the tubular lumina. Optimal conditions for dye uptake were found when the solutions were at pH 8 and experimental media were adjusted to this pH when necessary. Experiments were considered valid when the control kidneys displayed a maximal uptake of the dye within 30 to 60 minutes; such concentrations persisted for several hours under normal conditions. Observations were made during the course of each experiment as noted in the accompanying charts. A minimum of 6 tests was made with each experimental agent.

As noted in other studies on phenol red transport, a constant supply of oxygen was found necessary to maintain this process. Oxygen was supplied to the dishes containing the renal organs by means of hypodermic needles attached to an oxygen tank.

## MORPHOLOGICAL DEVELOPMENT OF THE PRONEPHROS

Although the morphological development of the pronephros has been described elsewhere (Jaffee, '54) certain aspects of pronephric development relative to the present discussion are noted here. The 6 mm *Rana pipiens* embryo becomes freed of its enveloping membranes and jelly coats on the 6th day of development. At this time the pronephros is structurally complete and is composed of three tubules which lead into a winding collecting tubule. The tubules and the collecting tubule are encased in a membranous pronephric capsule. The collecting tubule leads posteriorly into the pronephric duct, the latter emptying into the cloaca. Within the pronephric capsule the peritubular spaces are filled with sinusoidal capillaries derived from the posterior cardinal vein, and forming a renal portal system. There are no observable histological differences among the cells comprising the pronephric tubular complex in the 6 mm embryo (fig. 1).

The cells forming the pronephros display a definite differentiation from the 9th day onward. The tubules at that time are composed of high cuboidal cells which resemble the proximal tubule cells of other vertebrate kidneys (fig. 2), while the cells forming the collecting tubule appear similar to distal tubule cells (fig. 2). The observations in the present series indicate that the high cuboidal cells of the tubules are involved in phenol red transport.

## RESULTS

Phenol red transport was not detected at the 6 mm stage in a series of tests carried out on 4 separate groups of embryos. Extirpation of the pronephric anlage in *Rana sylvatica* (Swingle, '19) and *Amblystoma punctatum* (Howland, '21) was found to be followed by edema and death of the embryos when those embryos reached equivalent stages, so that the pronephros is presumably functional at this stage.

The observation that phenol red was not transported in the 6 mm embryo implies that other compounds which compete with phenol red for the transport mechanism (cf. competitive inhibition, below) are not excreted at this time. However, it would seem that the transport of ions concerned in water balance is taking place in the 6 mm embryo.

Examinations for dye transport were carried out daily on 6- to 11-day organisms. Marked phenol red transport was first observed in the pronephric tubules on the 11th day (11 mm tadpole); maximal dye uptake was then noted after 1 hour. Phenol red transport was thus noted at a stage in which tubular differentiation had been observed. In the original series of experiments, quoted above, slight traces of phenol red were noted in the tubules of stages between the 6 and 11 mm organism. A reinvestigation of this work together with a morphological study of these stages (7 to 10 mm) shows that a definite but submaximal dye uptake appears on the 9th day (9 mm). It is at this stage (9 mm) that cellular differentiation is first seen. These findings again strengthen the concept that dye transport and cellular differentiation appear concurrently.

As noted above maximal dye accumulation first appears in the pronephros of the 11 mm tadpole. Here, as in later stages, marked dye accumulation was noted within 15 minutes after placing the tubules in solutions undergoing oxygenation; maximal dye uptake was found by 30 minutes in most cases. The tubules of the pronephros (and mesonephros) were found to accumulate the dye in a uniform manner in the majority of the experiments, although variations in the ability of individual tubules (or among various kidneys) to perform this process were encountered. The tubule cells remained colorless during the dye transport process, but a yellow tint was seen in some instances when the transport process was suppressed by various inhibitors.

The pronephros was found present throughout the greater part of the three month larval period of *Rana pipiens* in the study of the normal development of this organ. Phenol red uptake could be demonstrated throughout this period, with the exceptions noted above. Uniform results were obtained in experiments carried out at various stages in the premetamorphic period indicating that the age of the individual was not a factor in the results obtained.

The morphological analysis further revealed that the mesonephros was structurally complete at the end of the first month. Examinations of phenol red uptake in the mesonephric tubules of month old tadpoles were positive, but tests on the forming tubules of three week specimens proved negative. The pro- and mesonephric kidneys of the *Rana pipiens* larvae thus appeared to function simultaneously for some two months of the larval life. Consistent results were obtained in experiments with the mesonephric tubules over a two month time interval.

Close agreement was found in the data gathered from experiments with both pronephros and mesonephros.

## *Aerobic metabolism*

The metabolic processes which are involved in the cellular transport mechanism appeared to be chiefly aerobic since dye transport took place only when an adequate supply of oxygen was provided. Cyanide inhibition of phenol red transport has been demonstrated in a number of renal forms and the effects of this agent on the transport system were examined on the kidneys of the developing organism. Complete inhibition of dye accumulation by the pronephric and mesonephric tubules was observed with M/100 KCN, partial inhibition was noted at M/500 KCN, while M/1000 KCN exerted only a slight effect (table 1). Complete reversibility of cyanide inhibition was found when the renal organs were

TABLE 1

*The effects of cyanide on phenol red transport*

|  | CONTROL | M/100 KCN | M/500 KCN | M/1000 KCN | M/2000 KCN |
|---|---|---|---|---|---|
| *15 minutes* |  |  |  |  |  |
| Pronephros | +++ | 0 | + | ++ | +++ |
| Mesonephros | +++ | 0 | + | ++ | +++ |
| *30 minutes* |  |  |  |  |  |
| Pronephros | ++++ | 0 | ++ | +++ | ++++ |
| Mesonephros | ++++ | 0 | ++ | ++ | ++++ |
|  |  | to control at 45 min. |  |  |  |
| *60 minutes* |  |  |  |  |  |
| Pronephros | ++++ | ++ | ++ | +++ | ++++ |
| Mesonephros | ++++ | ++ | ++ | +++ | ++++ |
| *90 minutes* |  |  |  |  |  |
| Pronephros | ++++ | ++++ | +++ | ++++ | ++++ |
| Mesonephros | ++++ | ++++ | +++ | ++++ | ++++ |

returned to the control solution. Varying the concentration of phenol red from 1 to 2 mg% had no bearing on the experimental results. Forster and Taggart have pointed out that the effective concentrations of cyanide in the flounder are probably lower than those observed, since hydrocyanic acid is weakened in the continuous oxygenation of the solution; this would seem to apply to the experiments with the tadpole as well as with those on the fish. Since cyanide is a well known inhibitor of the cytochrome oxidase system, the activity of this system in the cellular transport mechanism of the anuran pronephros and mesonephros is indicated.

Sodium azide, another known inhibitor of the cytochrome system, was also examined. Azide was found to inhibit dye uptake completely at M/50 in the pronephric and mesonephric tubules. Partial inhibition was obtained at M/100 while M/200 azide did not affect the transport process. The effects of azide were found to be reversible.

### *Oxidative phosphorylation*

The action of 2,4 dinitrophenol in suppressing the formation of pyrophosphates without affecting cellular oxidation has been demonstrated in the mammalian kidney by Cross. Taggart, Covo and Green ('49). Taggart and Forster ('50) have analyzed the effects of a series of nitrophenol compounds on the phenol red transport system in the isolated flounder tubules. They found that concentrations of 2,4 dinitrophenol which inhibited dye transport in the flounder tubules stimulated the respiration of the tubule cells and have concluded that cellular transport was partly dependent upon the utilization of phosphate bond energy. Some of these experiments have been repeated in the present series in order to investigate the oxidative phosphorylation process in the embryonic kidney. The effects of 2,4 dinitrophenol on the pronephros and mesonephros of the tadpole are illustrated in table 2. The accumulation of phenol red by the renal tubules was suppressed entirely by 2,4 dinitrophenol at M/10,000 with partial inhibition of this process being noted at M/50,000. Returning the tubules to the control medium was followed by a reversal of the inhibitory action of this compound. The concentrations of 2,4 dinitrophenol which were effective in suppressing phenol red transport in the tadpole were similar to those reported by Taggart and Forster. Oxidative phosphorylation thus appeared to be involved in the transport system of the pronephros and mesonephros of the developing anuran.

TABLE 2

*The effects of 2,4 dinitrophenol on phenol red transport*

| | CONTROL | M/10,000 DPN | M/10,000 DPN | M/50,000 DPN | M/100,000 DPN |
|---|---|---|---|---|---|
| *15 minutes* | | | | | |
| Pronephros | +++ | 0 | 0 | ++ | +++ |
| Mesonephros | +++ | 0 | 0 | ++ | +++ |
| *30 minutes* | | | | | |
| Pronephros | ++++ | 0 | 0 | +++ | ++++ |
| Mesonephros | ++++ | 0 | 0 | +++ | ++++ |
| | | | to control at 45 min. | | |
| *60 minutes* | | | | | |
| Pronephros | ++++ | 0 | ++ | +++ | ++++ |
| Mesonephros | ++++ | 0 | ++ | +++ | ++++ |
| *90 minutes* | | | | | |
| Pronephros | ++++ | 0 | ++++ | +++ | ++++ |
| Mesonephros | ++++ | 0 | ++++ | +++ | ++++ |

Among the nitrophenol compounds tested by Taggart and Forster was trinitrophenol (picric acid). Picrate was found to be an effective inhibitor of phenol red transport in the flounder tubules but did not stimulate respiration in the renal cells as in the case of 2,4 dinitrophenol, and Taggart and Forster have postulated that picrate did not inhibit aerobic phosphorylation but acted upon another component of the cellular transport system. In the tadpole kidneys picrate was found to suppress dye transport almost completely at M/40,000 and partial suppression was observed at M/80,000.

Reversibility of the effects of picrate were noted. These results are in the same range as those of Taggart and Forster.

### *Anaerobic glycolysis*

The finding that iodoacetate inhibited the accumulation of phenol red by the explanted tubules of the chick mesonephros has led Beck and Chambers ('35) to postulate that anaerobic glycolysis was involved in cellular transport. Iodoacetate has been shown to inhibit 1,3 diphosphoglyceraldehyde, a glycolytic enzyme (Adler, von Euler and Gunther, '38). Beck and Chambers noted that the effects of iodoacetate were counteracted by lactate. Forster and Taggart have found that the effects of iodoacetate were not counteracted by lactate in the renal tubules of the flounder, and have stated that anaerobic glycolysis was probably not involved in tubular transport. The work of Adler, von Euler, Gunther and Plass ('39) showing that iodoacetate inhibited a number of dehydrogenases involved in aerobic metabolism has been cited by Forster and Taggart in support of their view. The effects of iodoacetate and lactate were examined in the pronephros and mesonephros of *Rana pipiens* and some of the results obtained are summarized in table 3. Phenol red accumulation was completely suppressed at M/2000 iodoacetate. Pretreatment of the tubules with sodium lactate, followed by transferring them to an iodoacetate-lactate mixture, as recommended by Beck and Chambers, did not counteract the effects of iodoacetate. Placing the tubules in a lactate solution after exposure to iodoacetate was also ineffective. The effects of iodoacetate and lactate observed on the embryonic and larval anuran kidneys are in agreement with those of Forster and Taggart.

Examinations of the action of sodium fluoride on phenol red transport in the flounder tubules have been made by Forster and Taggart. Although fluoride is known as an inhibitor of enolase, a glycolytic enzyme (Warburg and Christian, '42), they found that fluoride was completely effective in inhibiting dye transport only at M/50 and that this agent was a powerful respiratory inhibitor of the tubule cells at that concentration. Forster and Taggart have stated that the action of fluoride, like iodoacetate, was primarily directed against aerobic metabolism. Fluoride was observed to block dye transport almost entirely at M/50 and exerted a partial inhibitory effect at M/100; M/200 fluoride had little effect on the transport process. These effects were found to be reversible.

TABLE 3

*The effects of iodoacetate and lactate on phenol red transport*

| | CONTROL | M/2000 $CH_2ICOOK$ | M/200 LACTATE | M/200 LACTATE |
|---|---|---|---|---|
| *15 minutes* | | | | |
| Pronephros | +++ | +++ | +++ | +++ |
| Mesonephros | ++ | ++ | +++ | ++ |
| *30 minutes* | | | | |
| Pronephros | ++++ | ++++ | ++++ | ++++ |
| Mesonephros | ++++ | ++ | ++++ | ++++ |
| | | | | 45 min: to M/2000 Iodoacetate + M/200 Lactate |
| *60 minutes* | | | | |
| Pronephros | ++++ | 0 | ++++ | ++++ |
| Mesonephros | ++++ | 0 | ++++ | ++++ |
| *90 minutes* | | | | |
| Pronephros | ++++ | 0 | ++++ | +++ |
| Mesonephros | ++++ | 0 | ++++ | +++ |
| | | | | to control |
| *120 minutes* | | | | |
| Pronephros | ++++ | 0 | ++++ | + |
| Mesonephros | ++++ | 0 | ++++ | + |
| *135 minutes* | | | | |
| Pronephros | ++++ | 0 | ++++ | ++ |
| Mesonephros | ++++ | 0 | ++++ | + |

### *Competitive inhibition*

The presence of a common transport mechanism in the renal tubules has been postulated by Smith, Goldring and Chasis ('38). They found that phenol red excretion was depressed in the human kidney when the plasma levels of diodrast and hippuran were raised. On the other hand, when the plasma level of phenol red was raised, the rate of excretion of diodrast and hippuran was depressed. Forster and Taggart reported that p-aminohippurate and penicillin, substances known to be excreted by the renal tubules, inhibited phenol red uptake in the isolated flounder tubules.

The addition of M/800 p-aminohippurate to the phenol red solutions was followed by an almost complete suppression of dye uptake by the tubules of the anuran pronephros and mesonephros. Reversibility of this effect was obtained by returning the tubules to the control medium. Partial inhibition of dye transport was noted with M/2000 p-aminohippurate while lower concentrations of this compound did not affect the transport process. The phenol red in the medium was in 1 mg % concentration (approximately M/35,000) in these experiments. When the phenol red concentrations were increased to 2 mg %, p-aminohippurate inhibited tubular transport only at M/200 concentration while partial suppression was found at M/400. M/800 p-aminohippurate had no effect on dye uptake. The effects of p-aminohippurate on phenol red transport are summarized in table 4.

Procaine penicillin G (Squibb) blocked phenol red uptake completely at M/600 and partially at M/1200 concentrations when the medium contained 1 mg % phenol red. The effective inhibitory concentration of penicillin was raised to M/300 in 2 mg % solutions of phenol red with partial inhibitory effects being found at M/600. The effects of uric acid on dye transport were examined since this compound is known to be excreted by the renal tubules of several forms. When uric acid was added to solutions containing 1 mg % phenol red, dye uptake was inhibited at M/680 uric acid, partial inhibition of the transport process was noted at M/1700, while M/3400 uric acid did not affect the transport process. In 2 mg % phenol red solutions M/340 uric acid was effective in inhibiting phenol red uptake with M/680 exerting a partial inhibitory effect.

The reversible inhibition of dye transport by p-aminohippurate, penicillin and uric acid in the pronephric tubules has indicated that these compounds are excreted and exert their effects by competing with phenol red for the transport mechanism. The broad range of inhibitory concentrations revealed by increasing the dye content of the solutions further supports this concept. The common transport mechanism described for other renal forms thus appears to be operative in the pronephros.

TABLE 4

*Reversible effects of P-aminohippurate (PAH) on phenol red transport*

| | CONTROL | PHENOL RED 1 MG % | | PHENOL RED 2 MG % | |
|---|---|---|---|---|---|
| | | M/800 PAH | M/2000 PAH | M/200 PAH | M/800 PAH |
| *15 minutes* | | | | | |
| Pronephros | +++ | 0 | + | + | ++ |
| Mesonephros | +++ | 0 | + | + | ++ |
| *30 minutes* | | | | | |
| Pronephros | ++++ | 0 | +++ | + | +++ |
| Mesonephros | ++++ | 0 | +++ | | +++ |
| | | to control at 45 min. | | | |
| *60 minutes* | | | | | |
| Pronephros | ++++ | + | +++ | + | ++++ |
| Mesonephros | ++++ | + | +++ | + | ++++ |
| *90 minutes* | | | | | |
| Pronephros | ++++ | ++++ | +++ | + | ++++ |
| Mesonephros | ++++ | ++++ | +++ | + | ++++ |

Creatinine is excreted by the renal tubules of teleosts but not by the tubules of the frog, turtles and some mammals (Smith, '51). The ability of the renal tubule of the primate to excrete creatinine has probably been acquired independently rather than inherited from the teleosts, according to Smith. The effect of creatinine on tubular excretion was examined in the pronephros. Creatinine did not affect dye transport in concentrations as high as 500 mg % (approximately M/20) and apparently is not excreted by the pronephric tubules. These findings in the embryonic kidney appear to support Smith's view.

## SUMMARY AND CONCLUSIONS

The cellular transport of phenol red heretofore demonstrated in the mesonephros and metanephros, has now also been shown in the pronephros. The data provided by the present experiments are in close agreement with the work of Forster and Taggart, and in the same range as the results obtained by Beyer, Painter and Wiebelhouse. It can be concluded, therefore, that the cellular transport mechanism is fundamentally similar in the pro-, meso-, and metanephric kidneys.

Cellular transport took place only when an adequate oxygen supply was present, indicating that this process was chiefly dependent upon aerobic metabolism. The role of dehydrogenases in these oxidative reactions was implied by the actions of iodoacetate and fluoride, and the effects of cyanide and azide suggested that the cytochrome oxidase system was operative in the transport process. The utilization of phosphate bond energy in cellular transport was indicated by the results obtained with dinitrophenol. Para-aminohippurate, penicillin and uric acid appeared to exert their effects by competing with phenol red for the transport mechanism.

The initiation of tubular transport in the developing organism was found to coincide with the differentiation of the cells comprising the pronephric tubular complex. The question of whether the origin of the transport mechanism can be related to the appearance or activation of any specific component of the renal cell will be considered in further investigations.

The renal organs of the Myxinoidea and Gymnophiona develop from the nephrogenic tissue as a series of structurally similar tubules extending along the body of the organism (Fraser, '50). The pro-, meso- and metanephric kidneys all develop from the nephrogenic tissue, thus displaying a common embryonic origin (ibid). These findings have led Fraser to postulate that the vertebrate excretory system is essentially a single organ, or holonephros. The almost identical responses of the anuran pronephros and mesonephros to the experimental agents tested and the evidence of the concurrent functioning of these organs, are in support of this view.

The author wishes to thank Dr. T. W. Torrey for his guidance and encouragement during the course of this work.

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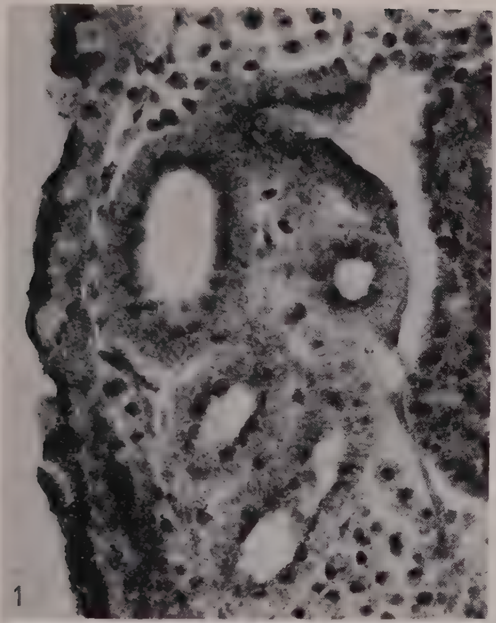
## PLATE 1

### EXPLANATION OF FIGURES

1 Cross section through pronephros of 7 mm stage. Histological differentiation among the cells comprising the pronephric tubular complex is not found in this stage and phenol red uptake was not noted at this time.

2 Cross section through the 11 mm tadpole. The tubular complex was found to be differentiated into high cuboidal cells of the tubules proper (T) and the lower cuboidal cells of the collecting tubule (CT). Phenol red uptake is well developed in this stage.



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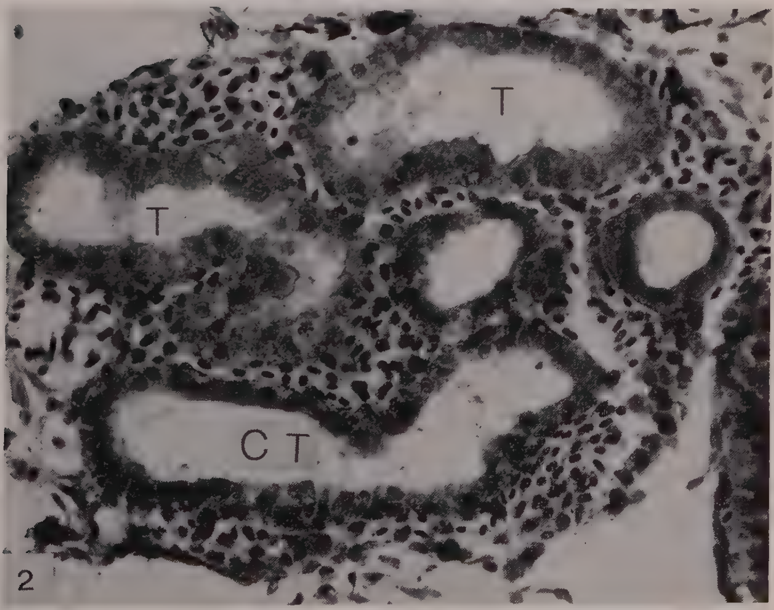

2

# ANALYSIS OF THE LUMINESCENT RESPONSE OF THE CTENOPHORE, MNEMIOPSIS LEIDYI, TO STIMULATION[1]

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THIRTEEN FIGURES

## INTRODUCTION

Except for the continuous production of light in bacteria and fungi, light emission in practically all living organisms results only on stimulation, either directly, or through nerves or from the action of hormones.

Until recently research on the light response from stimulation has been restricted to eye observation. With the development of various methods of recording light intensity, the time course of various luminescences has been measured, even when the duration of the light is no more than a very small fraction of a second. The various experiments made on a wide variety of forms have been discussed in the comprehensive reference monograph on bioluminescence by Harvey ('52).

The first quantitative measurement on any bioluminescent system changing with time was obtained by Amberson ('22) for the light decay curve of a mixture of crude Cypridina luciferin and luciferase extracts. He used a continuously moving film exposed directly to the light to be measured through a slit in the mixing vessel.

The first use of photocell-amplifier techniques for recording the luminescence of Cypridina extracts was by Harvey and

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[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

Snell ('30), quickly followed by application to the elaterid beetle, Pyrophorus (Harvey, '31), and the fire-fly (Harvey and Snell, '31; Snell, '32; Brown and King, '31). For all these studies a string-galvanometer was used as the recording instrument.

Although a number of later quantitative studies on the fire-fly flash and Cypridina luminescence have been made and despite the many quantitative studies on the mechanical and electrical responses of muscle and the electrical responses of nerve to stimulation, the luminescent response of various animals has been neglected.

In 1952, Nicol ('52a, b; '54a, b) published his work on light emission from the worm, Chaetopterus. Using photomultiplier tube and amplifier, with galvanometer or oscilloscope he made a study of factors concerned in regulation of the magnitude of the luminescent responses under various stimulating conditions, in presence of drugs and changes in the external medium. The extracellular luminescence of Chaetopterus, however, cannot be logically compared with the response of muscles.

The author had attempted to record flashes from Noctiluca and other dinoflagellates during the summer of 1952, but difficulties in obtaining and culturing the organisms delayed the work. In 1953 a plentiful supply of the ctenophore, Mnemiopsis leidyi at Woods Hole, Mass., led to the experiments reported in this paper.

In the meantime Nicol ('53, '54c) published his studies on the luminescence of isolated scales of polynoid worms using instruments similar to those employed in this investigation. He mainly observed the rhythmic luminescence controlled by the nervous system, but also included some single responses with their time characteristics, and the facilitation phenomena observed in flashes following high frequency (above one per second) stimuli. Both polynoid worms and ctenophores respond to stimulation with a light emission that is remarkably similar, despite their morphological differences and lack of relationship. Their resemblance to muscle response is quite remarkable.

Mnemiopsis has turned out to be an ideal organism for investigation because of its availability, ease of handling and lack of a diurnal cycle in luminescence, although a bright light will temporarily inhibit the luminescence. Much work of a non-quantitative nature has been carried out on the luminescence of ctenophores since the early studies of Allman (1862) and Panceri (1872). Histology of the meridional canals has been investigated by Hertwig (1880), Chun (1880) and Dahlgren ('16). The emission of light resulting from various types of stimulation has been studied by Peters ('05), Moore ('24, '25b) and Harvey ('21). The effect of oxygen tension on light production of whole animals and extracted juice has been investigated by Harvey ('26), Harvey and Korr ('38) and Chase ('41). The inhibition of ctenophore luminescence in the light and its return in the dark has been observed by Allman (1862), Panceri (1872), Peters ('05), Moore ('24, '25a, '26), Heymans and Moore ('25), Harvey ('21, '25) and many others. Biochemical studies of ctenophore luminescence have been made by Harvey ('21, '26, '49) and Moore ('24, '25a), but no quantitative record of the luminescent response has been published prior to this work.

## MATERIAL AND METHODS

The collected specimens of *Mnemiopsis leidyi,* Agassiz, were placed in large aquaria with very slowly running sea water, where they keep well for as long as 10 days; but for quantitative experiments fresh animals collected within three days were always used. Upon stimulation luminescence appears along each meridional canal underlying the 8 rows of ciliary combs or paddle plates. Depending on the type of experiment, a whole meridional canal isolated from the animal or a small portion of meridional canal containing one to 4 paddle plates and a small amount of surrounding tissue was employed.

To observe the luminous responses from a small section of luminous canal the piece isolated from the animal was confined in a lucite chamber specially designed for this purpose. As shown in figure 1 A, it consisted of a specimen pool and two electrode pools. The former was designed to keep the specimen in a confined space with a desired orientation. The latter was shaped so that (1) there would be the least amount of resistance to the flow of stimulating current; (2) the current density would be great in the specimen pool by narrowing its entrance; (3) the effect of any by-products around the electrodes caused by current flow might be minimized by a large volume of sea water in the electrode pool. This chamber was then fitted into a permanent moulding of an electrode assembly which holds 8 adjustable Ag-AgCl electrodes, of which two were ordinarily used for stimulation. A light-tight box was employed to house the specimen and the electrodes, and a photomultiplier tube (RCA Type 1P21) was attached to the bottom of this box for flash detection.

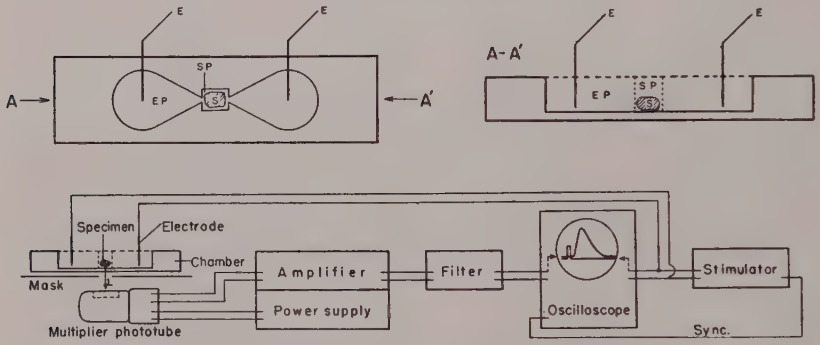

Fig. 1 A Top and cross-section side views of the chamber employed for work with a small section of a meridional canal. E, stimulating electrodes; S, specimen; SP, specimen pool; EP, electrode pool.

B Basic arrangement of apparatus for stimulating a small piece of a meridional canal and simultaneous recording of the luminous response and the stimulus signal.

The light measuring device mainly used in this study had a power source of negative D.C. voltage applied to the cathode and dynodes of a photomultiplier tube. This voltage was stabilized to 0.1% when the A.C. line voltage fluctuated as much as 10% in either direction, and was free from A.C.

ripple. The sensitivity of the photomultiplier tube could be varied over 5 decades of light intensity to be read on full scale by changing the voltage across the phototube in 9 steps from a maximum of — 1000 volts to a minimum of about — 250 volts, with constant stability at all voltages. The signal obtained from the anode of the photomultiplier was fed into a D.C. amplifier circuit within the unit.

The output of this amplifier was in turn connected to one beam of a dual-beam cathode ray oscillograph (DuMont Type 322) through a low-pass filter consisting of a constant k section, an m-derived middle section and two halves of m-derived end sections. The filter had a sharp cut-off frequency at 30 cycles in order to eliminate photocell noise which shows up in wide bands on the oscilloscope screen if not filtered.

An electronic stimulator of a D.C. square wave generator type (Grass Type IIIB) and having independent parameters of voltage, duration, frequency and external synchronization delay was used to give controllable and known stimuli to the specimen. The exact nature of the stimulus was simultaneously recorded with the response by feeding the stimulation signal into the second beam of the cathode ray oscillograph and using a common sweep for both beams (fig. 1 B). A Leica camera or a movie camera with a rapidly changeable single frame exposures was used for recording from a long persistent oscillograph screen. Whenever necessary the temperature was controlled by the use of a stainless steel water jacket, with a double glass bottom to allow light to pass through.

## RESULTS

### *Response to a single stimulus*

### Multiple response

As well known to many who have observed Mnemiopsis closely, the whole animal or an isolated meridional canal will respond with a greenish luminescence when stimulated by jarring the container, touching the specimen, or passing an electric current through it. This response, although it may appear to be a continuous single flash, is actually a series of flashes when observed on the cathode ray oscilloscope screen. When only a localized region of such a preparation is observed through a small opening, a single stimulation will usually result in a series of repetitive flashes (fig. 2 A). In this respect the response resembles the luminous responses of the polynoid elytrum reported by Nicol ('53, '54c). However, responses of the Mnemiopsis canal differ from those of a polynoid elytrum in that they are neither as regular nor as prolonged. The frequency of repetition in Mnemiopsis ranges from 5 to 12 per second as compared with an average of one to 9 per second in polynoid worms. The occurrence of any frequency or combination of frequencies within the extremes for any given single stimulus is purely random in Mnemiopsis. In many cases the series following a single shock is divided into irregular groups of flashes, each group containing one to 10 individual flashes. The number of flashes within a group and the time interval between the groups also do not have regularity. In all cases the whole series may be over within several seconds. The intensity of each flash is also unpredictable, except that the first few flashes within a subgroup usually tend to increase in magnitude. No gradual decrease of light level of each flash marks the end of a series of responses after a single stimulus. Using a single ctenophore meridian, a series of responses will not be obtained if stimulated too frequently, but a recovery period of about one minute must be allowed to again obtain a series of flashes.

## Single responses

A small piece of meridional canal containing only a few paddle plates, when stimulated electrically will give a very faint luminescent response. The principal characteristic of the response from a short piece of canal is that it gives off only one flash when stimulated by one electrical pulse, and that the light level and the time course of such a flash is to some degree repeatable under the same conditions. Furthermore the stimuli can be given as frequently as desired up to

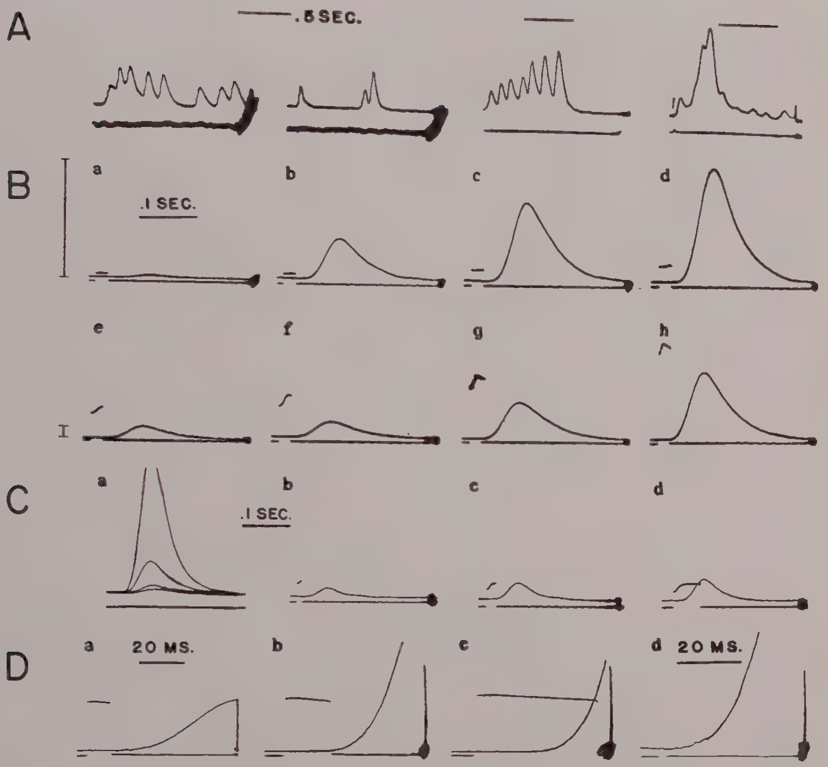

Fig. 2 A Actual records of 4 multiple luminous responses from a whole meridional canal, each after a single stimulus. The recording was localized to a small portion of a whole canal by placing a mask with a single slit between the canal and the photocell. Horizontal bars indicate 0.5 second.

B A series of luminous responses from a small meridional canal section as the voltage of a stimulus with a fixed duration is varied. The sensitivity of the light detector for records a–d is 10 times that for e–h as indicated by vertical bars representing one light unit.

C Two series of responses from small sections of canal as the duration of stimulation is varied at a fixed voltage. (a) shows multiple exposure records containing responses in the increasing order to 1, 2, 5 and 10 msec. at 128 volts. (b–d) shows a series of responses to stimulations of 10, 20 and 50 msec. (indicated by lower line) at 64 volts. Note in (a) the constancy of the flash duration even when the intensity of the flash response increases. A time scale for a–d is given as a bar.

D a–c. A series of records of latent period measurements as the duration of the stimulation is varied from 10 to 50 msec. The gain of the upper beam recording light intensity is greatly increased to enlarge the initial phases of the response. (a–c) have the same time scale as indicated by horizontal bar in (a). (d) shows the shortest latent period recorded. The stimulus was 2 msec. at 128 volts, with time scale indicated by horizontal bar.

the fusion frequency of the luminescent response without missing a response, whereas in larger pieces a response may be omitted, more or less irregularly, when stimulated faster than 4 per minute. The variation in behavior obtained when using such small pieces will be described below.

*Variation in voltage of stimulus.* The light intensity of the response resulting from a single stimulus varies with the voltage of the stimulating pulse. As the voltage of stimulation is increased gradually, with the duration of the pulse at a constant value, the response will not appear until a certain value of stimulating voltage is reached. This value is not less than 10 volts at 20 milliseconds (msec.) with the arrangement of electrodes previously described. With stimulating voltages above the threshold value, the light level of each response gradually increases as the stimulating voltage is increased (fig. 2 B). This positive correlation between the peak intensity of light response and the stimulating voltage is quite analogous to the response of a muscle fiber bundle when stimulated directly. Because of the complications connected with fatigue phenomena, the point of maximal response has not been definitely determined. A similar correlation between stimulating voltage and the light level of response occurs at both lower and higher temperatures, ranging from 7 to 35°C. The correlation is much more marked at high than at low temperatures.

*Variation in duration of stimulus.* As is the case with the change in voltage for stimulation, there is a definite correlation between the duration of the stimulating pulse and the intensity of luminescent response (fig. 2 C). Gradual increase of duration above a threshold value causes a continuous increase in light level of response (fig. 2 C). The effect of prolonged pulse duration has not been studied in this investigation because of limitations in the stimulator. Stimulation with relatively long durations (20 msec. or longer) often causes very rapid fatigue, making regular repeatable responses difficult to obtain. Over a wide range of temperature (7–35°), the intensity of the response increased with increase of stimulus pulse duration, just as observed at room temperature, 22°.

*Analysis of time course of single flashes.* It is of interest to consider some characteristics of the time course of single flashes, using the extensive data obtained from the records. Just as a muscle twitch is divided into latent period, contraction phase and relaxation phase, so the time course of a single flash response can be divided into latent period, rise phase and decay phase of flash intensity (fig. 3).

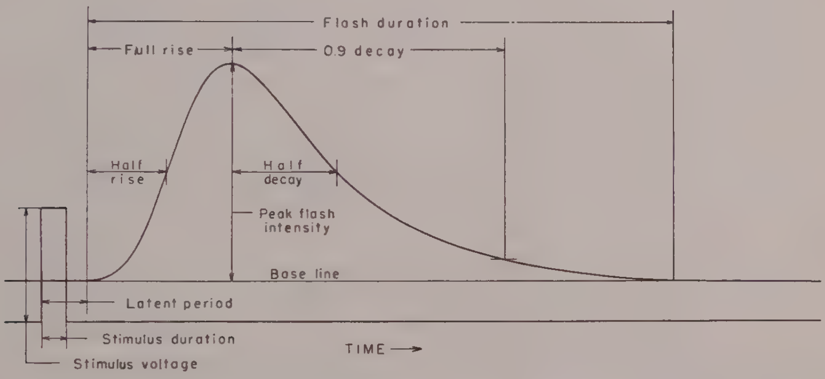

Fig. 3 Trace of a typical single flash at room temperature (23°) with designations for time analysis of the stimulus signal and the flash response.

*Latent period.* The latent period measured from records of small sections of canal varies greatly with the conditions of the measurement (fig. 2 D). The shortest and seemingly the actual latent period of the light producing cells lies between 4.5 and 10 msec., when stimulated with a strong pulse of short duration (1 to 5 msec.). Variation of voltage and duration results in marked change of the latent period. The weaker stimuli cause a longer latent period. The same kind of relationship has been observed in the sartorius muscle of Rana pipiens by Sandow ('44). In all the luminous records obtained, the latent period ranged between 4.5 to 38 msec. Nicol ('35) has reported a latent period for single flashes in Acholoë of 18 to 20 msec. and in Polynoë of 19 msec., probably at room temperature, and under one condition of observation. The effect of temperature is such as to increase the latent period when the temperature is lowered, and to decrease it when the temperature is raised. The same kind of effect has been observed by Sandow ('47) in frog muscles.

*Shape of the flash curve.* Measurements from about 250 single flashes have been made on half rise time, full rise time to peak intensity, half decay time and nine-tenths decay time under various experimental conditions. At room temperature, or at any other given temperature the intensity may vary, but the time course of a single flash remains fairly constant, within the normal distribution range, when the voltage and the duration of the stimulating pulse are changed. A similar relation has been observed in the case of the firefly flash by Snell ('31, '32) and by Brown and King ('31); although intensity varied, the time course of the flash was relatively constant. In muscle it has been reported that the form of the twitch remains constant under various conditions, except when the temperature changes (Gilson et al., '47). Firefly flash records (Brown and King, '31; Snell, '31, '32; Alexander, '43) are more or less symmetrical, whereas the ctenophore flash is somewhat skewed at room temperature. In Mnemiopsis, after a varying latent period the mean values and the standard deviations of means show that the flash reaches half of its maximum intensity in 34.6 ± 0.64 msec., and peak intensity in 59.9 ± 0.61 msec. It decays to half intensity in 47.8 ± 0.76 msec. and to nine-tenths intensity in 114.3 ± 1.5 msec. at a room temperature of 21.0°–23.5° (fig. 3).

As shown in figure 3, the response starts with a slow rise and within a very short time a rather fast rate of ascent is reached. This continues to near the maximum intensity where it is slowed to zero. The decay phase has a slow rate of fall near the peak and the rest of the decrease in intensity is logarithmic in nature, giving a straight line when the logarithm of light intensity is plotted against time (fig. 5). A comparison of some other response curves with that of Mnemiopsis is presented in table 1, where time is given in milliseconds.

TABLE 1

*Time course of response curves in various systems*

| SYSTEM | TEMPERATURE | HALF RISE TIME | FULL RISE TIME | HALF FALL TIME | FULL FALL TIME | INVESTIGATOR |
|---|---|---|---|---|---|---|
| | °C. | *msec.* | *msec.* | *msec.* | *msec.* | |
| Mnemiopsis flash | 21–23.5 | 34.6 | 59.9 | 47.8 | 230[2] | the author |
| Acholoë flash | Room? | .. | 18–23 | 11–19 | 45–80 | Nicol, '53 |
| Polynoë flash | Room? | .. | 26–30 | 17 | 108 | Nicol, '53 |
| Photuris pennsylvanica flash | 24 | .. | 70[1] | .. | 70[1] | Snell, '32 |
| | 23 | .. | 60 | .. | 90 | Brown and King, '31 |
| Photinus pyralis flash | 26 | 30[1] | 55[1] | 20[1] | 70[1] | Alexander, '43 |
| Achromobacter fischeri ''flash'' | 21.6 | .. | 340 | .. | 2510 | Schoepfle, '40 |
| Rat, m. triceps surae contraction | 36.4 (Body) | .. | 14.2 | 21.5 | .. | Walker, '49 |
| Frog, m. gastrocnemius contraction | 27.5 | .. | 27[1] | 54[1] | .. | Gilson et al., '47 |

[1] Measured by the author from published records.

[2] Nine-tenths decay time is 114.3 msec.

*Effect of temperature on the flash curve.* The effect of temperature on the time course of the flash is summarized in table 2, over the range of 12° to 35°. At temperatures higher than 35° the luminescence disappears rapidly and at 39° no light can be detected. If the tissue remains at 39° for two minutes, the cessation of luminescence is permanent, and cannot be reversed by a return to the room temperature. At lower temperatures the decrease of light intensity can be reversed by bringing the tissue back to room temperature. Table 3 shows the effects of temperature in other systems. Unlike some observations on skeletal muscle twitches, change in temperature in ctenophore flashes causes more marked changes in the decay phase than the rise phase. Both the time required to reach the peak and the time to return to darkness is prolonged as the temperature is decreased, while increased temperature causes shortening of both rise and decay. Figure 4 shows a comparison of time course of some typical response curves at different temperatures. At higher temperature, mainly as a result of shortening of the decay time, the flash curve becomes more symmetrical than at other temperatures. Such flash curves are similar to the firefly flashes at 23–24°. At all temperatures the intensity-time decay curve of Mnemiopsis is logarithmic, as shown in figure 5.

These 5 plots show very effectively the variation in the rate of decay at different temperatures. The rate of rise is either not or little effected by the temperature, except for the region near the peak, which causes a difference in the full rise time at different temperatures. Since the slope of these straight lines represents the rate of the luminous reaction at different temperatures, the logarithms of these slopes can be plotted against the reciprocal of the absolute temperature for an Arrhenius analysis. The resulting points give an approximately straight line (fig. 6). The temperature characteristic or activation energy, $\mu$, calculated from the equation

$$\ln k_1/k_2 = \mu/R(1/T_1 - 1/T_2)$$

where k is rate of reaction, T is absolute temperature, and R is the gas constant, 1.986, is about 12,000 calories.

## Response to multiple stimuli

*Summation of subthreshold stimuli.* When a small piece of Mnemiopsis canal is stimulated with a single shock, whenever the voltage, or the duration, or a combination of these two is less than a certain value, no luminous response occurs. However, by repeating such subthreshold stimuli two or more times it is possible to obtain a flash from the same piece. The number of subliminal stimuli required to excite the system to luminescence depends on the voltage, duration and the frequency of repetition of stimuli, the weaker intensity and the slower frequency always necessitating a greater number.

TABLE 2

*Effects of temperature on the time course of luminescent response in Mnemiopsis*

| TEMPERATURE | HALF RISE TIME | FULL RISE TIME | HALF DECAY TIME | 0.9 DECAY TIME |
|---|---|---|---|---|
| °C. | msec. | msec. | msec. | msec. |
| 12–15 | 45.22 ± 1.47[1] | 87.37 ± 3.66 | 105.48 ± 8.18 | 253.84 ± 19.79 |
| 17.5–18.5 | 47.33 ± 2.02 | 80.93 ± 4.05 | 69.47 ± 3.22 | 163.00 ± 9.96 |
| 21.0–23.5 | 34.66 ± 0.64 | 59.86 ± 0.61 | 47.76 ± 0.76 | 114.30 ± 1.50 |
| 25.6–26.8 | 30.38 ± 0.33 | 52.07 ± 0.43 | 36.67 ± 0.64 | 93.11 ± 1.26 |
| 31–35 | 29.08 ± 0.33 | 49.66 ± 0.53 | 28.51 ± 0.38 | 65.76 ± 1.27 |

[1] Standard deviation of the means.

TABLE 3

*Effects of temperature on the time course of the muscle twitch*
*(From the data on m. triceps surae of rat, Walker, '49)*

| EXPERIMENT | TEMPERATURE | FULL RISE TIME | HALF FALL TIME |
|---|---|---|---|
| | °C. | msec. | msec. |
| Whole body cooling | rectal temp. | | |
| | 22.0 | 74.2 | 92.8 |
| | 26.1 | 35.6 | 38 |
| (control) | 36.4 | 14.2 | 21.5 |
| Whole body cooling | muscle temp. | | |
| | 24.5 | 78 | 97 |
| | 28.8 | 53 | 70 |
| | 31.5 | 32 | 35 |
| (control) | 37.0 | 17 | 21 |

*Summation of flashes.* If two stimuli above the threshold intensity are given in rapid succession so that the second stimulus is delivered before the complete flash response is over, the second flash will be added to the first one. The

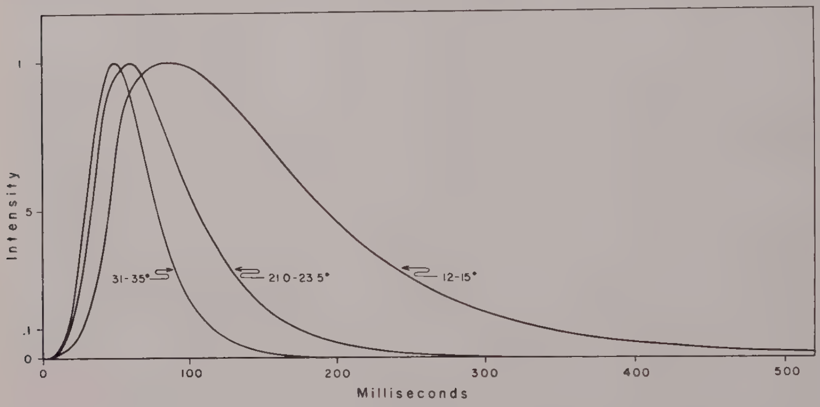

Fig. 4 A comparison of the time course of flash responses at different temperatures showing typical shapes on the same time scale. Peaks have been brought to the same height of 1.

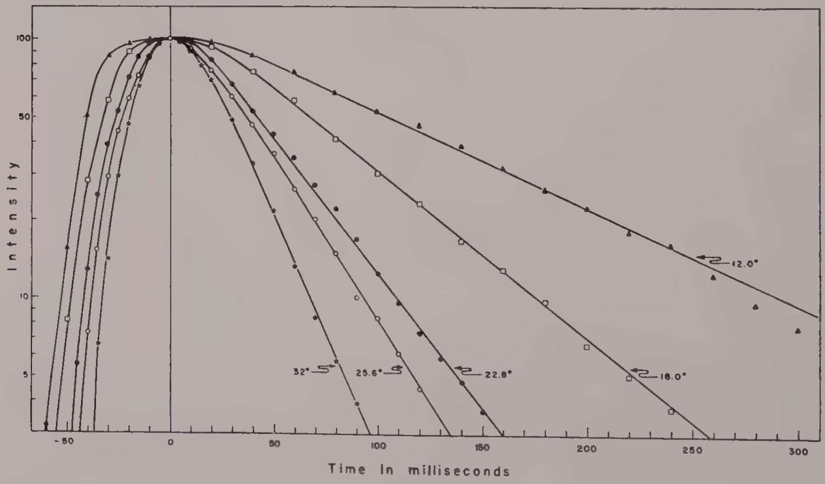

Fig. 5 Logarithms of light intensity plotted against time at different temperatures. Note the straight line characeristic of the decay phase and the effect of temperature on the rate of decay. The rate of rise is little effected by temperature changes. Flash peaks at different temperatures have been designated 100 for ready comparison and time is measured from this point.

records obtained at room temperature by giving two or more such stimuli at different frequencies are shown in figure 7. Although there are no comparable responses reported in other luminous systems, the records are strikingly analogous to those obtained from skeletal muscle. At a room temperature of 25°, repeated stimulation at 10 per second causes the second and any consecutive flashes to appear when the previous flash is well under way toward completion. Stimulation at 30 per second produces almost one continuous flash without showing any peaks or shoulders. Above 30 stimuli per second the intensity of the response increases with an increase in the number of stimuli. These results are in agreement with the time analysis of the flash previously described at this temperature in that the flash duration from the start to nine-tenths decay is about 150 msec. and the time to maximum intensity is about 53 msec. Shocks having frequencies between 10 and 30 give flashes with partial peaks or shoulders, depending on the repetition rate. The relation of intensity to the number of stimuli generally holds in these summated flashes. However, general fatigue of the specimen and some

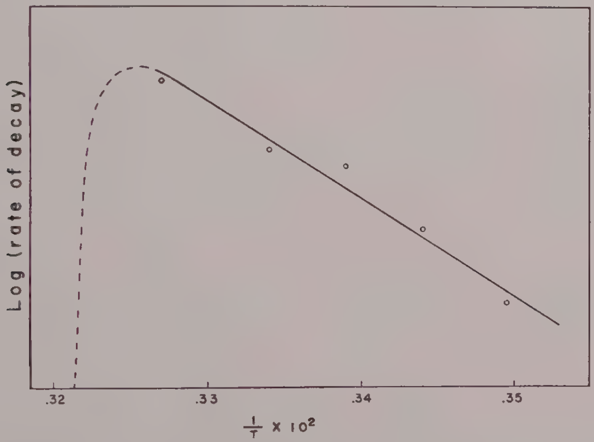

Fig. 6 Relation of the rate of flash decay to temperature as indicated by an Arrhenius plot. Ordinates: logarithms of the rate constant of flash decay; abscisae: reciprocals of the absolute temperature.

onset of staircase phenomena (treppe) or facilitation, which tend to make consecutive flashes larger than the previous ones, complicate the picture so much that any quantitative analysis cannot be made with the present set of data. At temperatures other than 25° the change in the time course of a single flash is reflected in the shapes of the summated response. In figure 8, the summation of flashes produced by stimulation frequencies of 10 per second and 20 per second

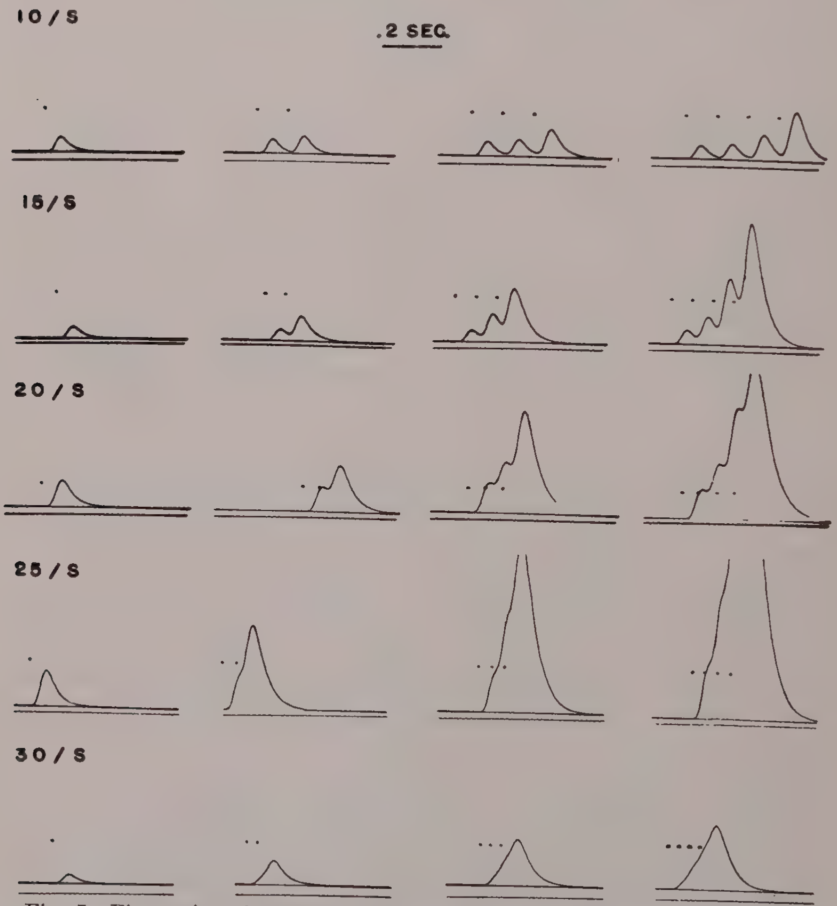

Fig. 7 Five series of records of summated flashes at different frequencies of stimulation: 10, 15, 20, 25 and 30 stimuli per second. 1, 2, 3 and 4 stimuli were given at each frequency.

are compared at different temperatures. It may be added here that at higher or lower temperatures than the optimum temperature (21.5° according to Peters, '05), the responses to repeated stimulation have some tendency to be slightly irregular.

*Treppe or facilitation.* In stimulating a small piece of Mnemiopsis canal with single shocks spaced at intervals longer than what corresponds to a complete flash, the staircase phenomenon (treppe) or facilitation is regularly observed. Each successive separate flash intensity is slightly greater than the one produced by the preceding shock, although the strength of the stimulus remains the same (fig. 9). It is not known whether the increased light intensity of successive responses is due to an effect in nerve which may properly be called facilitation, or in luminous cells, where the word "treppe" should apply. Treppe or facilitation is also observed in responses where summation of flash takes place. In this case each consecutive flash has a peak of greater height from the point of origin as well as from the base line, than the previous one. Of course, the height from the base line will be

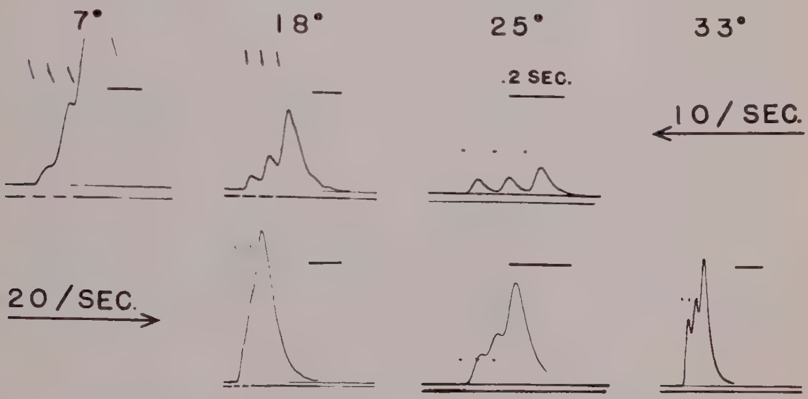

Fig. 8 Comparison of the summation of flashes at different temperatures. Three stimuli were given at the rate of 10 and 20 stimuli per second. Note the different degree of dips or shoulders in each case.

the sum of the effects of treppe or facilitation and summation in such cases. Such treppe or facilitation is observed in summated responses to faster frequency of stimulation up to the fusion frequency (fig. 10).

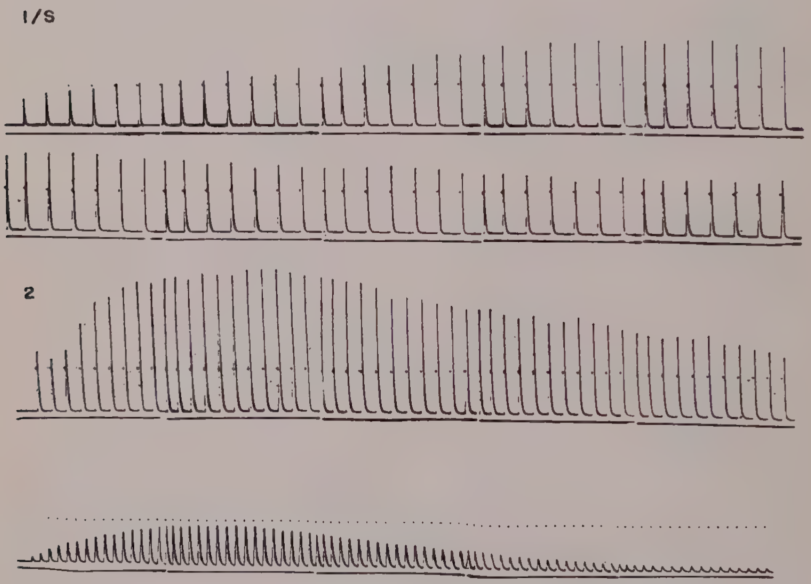

Fig. 9 Flash responses to repeated stimulation at frequencies of 1, 2 and 3 stimuli per second. The intensity and the duration of the stimuli are uniform. Note the staircase (treppe) or facilitation phenomena and the onset of fatigue.

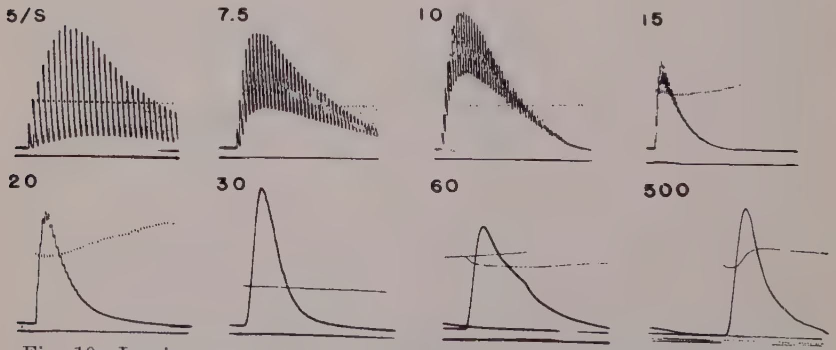

Fig. 10 Luminous responses to increasing frequency of stimulation, showing incomplete and complete ''tetani'' and relatively rapid fatigue. Numbers indicate the rate of stimulation per second.

*"Tetanus."* Phenomena comparable to tetanus of muscle can also be observed in the luminous system of Mnemiopsis. When stimuli having more rapid frequencies than a flash duration are continuously applied, the level of light intensity will remain raised above the base line for some time. Figure 10 shows such "tetani" at different frequencies of stimulation. At low frequencies, although not below 5 per second, each individual flash is very distinct and the decay phase continues almost to the base line. The individual flashes become less distinct as the repetition rate is increased. Finally, the whole response will become a smooth curve as each flash is completely fused with another. This occurs when stimulation frequency is about 30 per second, and conforms to the previously described observations on summation at the same frequency. Above this fusion frequency all responses to continuously repeated shocks are simple smooth curves. These responses to high frequency stimulation rise to a peak intensity very rapidly and immediately start to fatigue without producing any plateau such as is usually observed in muscular tetanus.

*Fatigue.* When the luminous system of Mnemiopsis is stimulated continuously by repeated shocks, fatigue is expressed as a fall of light intensity level (figs. 9, 10). As previously mentioned, the onset of fatigue is rapid in this system and is so marked that no flat plateau of constant light level is to be observed. The development of fatigue appears to depend chiefly on the frequency of stimulation. At as slow a rate of stimulation as one per second, the maximum intensity developed in a treppe will decrease to one-half in about 120 stimulations, but at the end of 440 stimulations the flash response is still detectable (fig. 11). At a stimulation rate of 500 per second, the time from the start of the response to the completely fatigued state is only 0.6 second. The strength of stimuli also effects the fatigue in the system. The stronger stimuli cause faster fatigue. This is particularly noticeable when the duration of each stimulus is prolonged.

Fatigue can also be seen in the preparation of a whole canal giving multiple responses (fig. 12 A). In such preparations fatigue sets in more easily since each response induces multiple flashes, thus causing complete fatigue to occur after only 10 or so shocks, even though they are separated by about 30 seconds. A completely fatigued small piece cannot recover by resting . Some permanent degeneration process

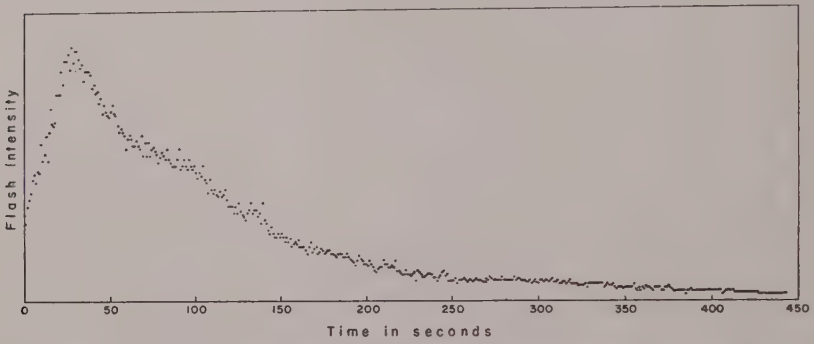

Fig. 11 Intensity of flash response of a small piece of canal to repeated stimulation at the rate of one per second, showing phenomena of staircase (treppe) or facilitation and general fatigue. Each point represents a response to a single stimulus.

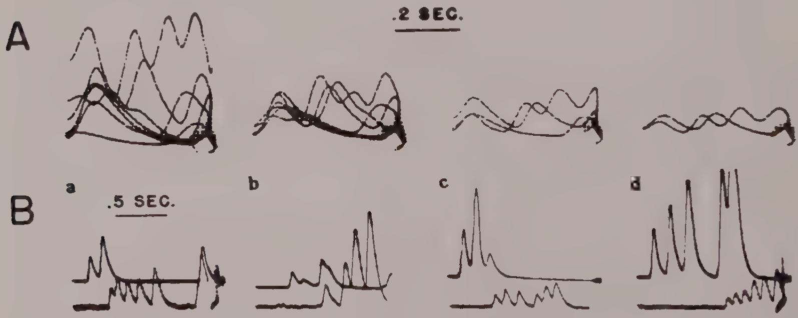

Fig. 12 A A series of records showing fatigue of multiple responses of a whole canal to 4 single stimuli given after successive intervals of 30 seconds. Light recording was localized by use of a mask with a slit. The oscilloscope was triggered internally in these records.

B Records taken by means of two separate photomultiplier detectors, showing measurements of conduction velocity of the luminous excitation along a whole canal. (a) and (d) are of fast and slow conduction respectively; (b) and (c) have average speeds as measured by the time interval between appearance of light in upper and lower line. Note the multiple nature of the response to single stimulus in each case.

seems to set in which affects the whole tissue after so many stimuli. In a complete canal preparation, the luminescence may recover in favorable specimens if permitted to rest some time. Nicol ('53) has reported that no recovery from a completely exhausted polynoid scale could be observed. The fatigue of a luminescent response in a whole Mnemiopsis has been studied by Peters ('05), who found it difficult to exhaust the luminous reaction.

### Conduction of luminescent excitation

It is a well known fact that in a whole ctenophore or in an isolated meridional canal the luminescent response, caused by any form of moderately strong stimulation, is not restricted to a localized portion of a canal but is conducted to other parts of the same canal. Unlike the rhythmic waves of excitation affecting the movement of paddle plates in Mnemiopsis which proceed from the aboral end of the animal and pass orally along the 8 meridional rows, the wave of luminescence can move in either direction. About 70 measurements have been made on the speed of conduction of the luminous response by two photomultiplier detectors. Figure 12 B shows some of the typical measurement records. At room temperature of 22.6°–25.0° the rate varied from 4.7 to 26,6 cm per second, with an average and the standard deviation of the means of $13.71 \pm 0.74$ cm per second. The speed of conduction is the same in either direction. This value is the same order of magnitude as the conduction speed of other excitable systems in coelenterates (see Prosser et al., '50, p. 783). Parker's value ('20) on the spread of a luminous wave in Renilla is 7.4 cm per second, while Panceri (1872) measured the velocity of propagation of luminous waves in Pennatula and found an average value of 5 cm per second, both at an undisclosed temperature.

### Action potentials simultaneous with luminescence

When recording electrodes are placed along a row of paddle plates on a meridional canal, potential changes can be observed both with and without a luminescent response. In 92 out of 94 records a luminescence was accompanied by bursts of action potential, which were observed either as a

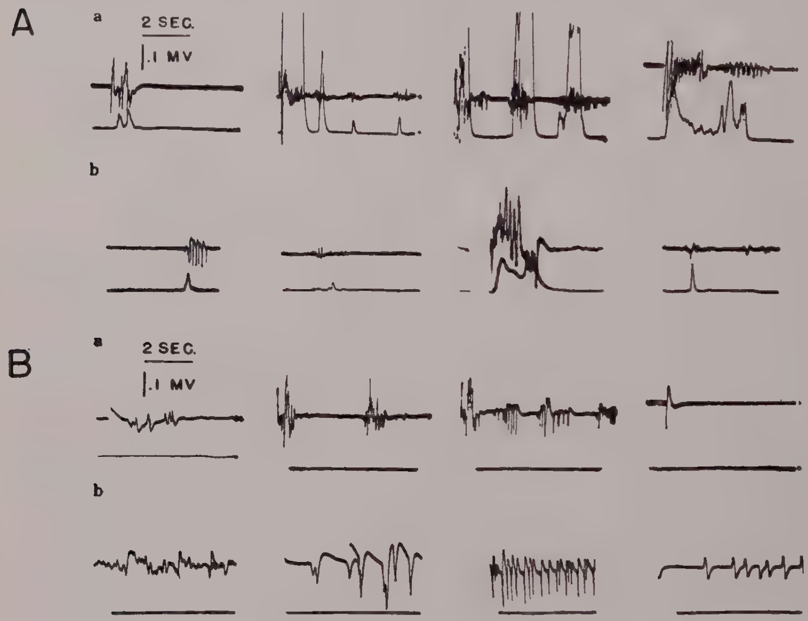

Fig. 13 A Records of potential changes (upper line, recorded through an A.C. preamplifier) simultaneous with the luminous response (lower line). a, responses resulting from electrical stimulation; b, spontaneous responses, or on slight agitation of the preparation. The potential calibration is shown as a vertical bar.

B Records of potential changes (upper line) along the meridional canal without luminous response (lower line). a, potential changes after electrical stimulation; b, spontaneous potential changes observed along the canal. The last record on the series (a) shows the stimulus artifact without a potential change or a light response.

result from slight jarring or injury (fig. 13 A). The largest electrical variation observed was about 600 μvolts.

The bursts of action potential occurring with the luminescent response have fast frequencies up to 100 cycles per second. Various other types of potential changes, some slow and some fast, also register spontaneously without luminescent response (fig. 13 B). Action potentials do not occur in other parts of the animal when resting or even when the oral lobe tissue containing many muscle fibers is stimulated. No potential changes with the same time relations as a single luminous flash were observed.

## DISCUSSION

An important characteristic of the luminous response of Mnemiopsis is the repetitive flashes of light which appear when a long piece of canal is stimulated. Polynoid scales respond in a similar manner with repetitive flashes (Nicol, '53). The fact that the repetitive response after a single stimulus disappears when a small piece of meridional canal is studied suggests the possibility of interference with a complicated nerve control mechanism responsible for repetitive response in the larger canal or the whole animal.

It is probable that when a small section of meridian is stimulated, the photogenic cells are excited directly rather than through nerves. This view is substantiated by the observation that in larger preparations where nervous influences are undoubtedly present, the refractory period is very long and varies greatly, whereas in a small piece the refractory period is less than the interval between the stimuli which will cause the fusion of responses. Moreover, the general behavior of the light response appears quite analogous to the response of directly stimulated muscle fibers. On the other hand, the great variability of latent period from preparation to preparation even in short pieces of canal suggests that nervous elements might receive the stimulus and then excite the photogenic cells. The marked facilitation also points to nerve stimulation, since the nervous nature of facilitation connected with muscular response in coelenterates has been well demonstrated in sea anemones by Pantin ('35a, b, c, d) and in syphomedusae by Bullock ('43). The Mnemiopsis records also bear a strong resemblance to facilitation observed in kymograph records of muscle contraction in syphomedusae reported by Bullock ('43). The question whether the phenomena observed in Mnemiopsis is a "staircase (or treppe)," involving only the photogenic cells directly, or a facilitation taking place through the nervous system cannot be answered until critical tests on direct or indirect stimulation, involving the use of preparation under the influence of drugs are made.

The phenomenon of auto-fatigue (decrease in size of repetitive flashes after a single stimulus) observed in the polynoid scale by Nicol ('53) is not observed in Mnemiopsis preparations, either small or large. In Mnemiopsis fatigue can only be observed, in small sections, after repeated stimulation. The dependence of the rate of fatigue on the frequency of stimulation is further demonstrated by the amount of total light produced for each frequency until complete fatigue is reached. It has been found that the total light produced is greater when the stimulating frequency is slower. As the frequency is increased, the total light becomes less to a certain frequency, above which point no more decrease in total light is observed. This probably indicates that photogenic material is continuously being produced. The fact that it takes only a short time for a relatively fatigued tissue to recover almost completely, ready for another series of repeated stimuli, and that the whole animals are difficult to exhaust in their capacity for light production by continuous agitation (Peters, '05) also supports this view.

In most bioluminescent systems fatigue after repetitive stimuli is very rapid. Fatigue is one of the difficulties encountered using any luminous organism for quantitative studies. However, in Mnemiopsis at a slow rate of stimulation, the constancy of light intensity in each preparation does not vary too much. At a stimulation frequency of one per second, 100 or more responses can be obtained before fatigue brings the light intensity to half value. And at slower frequencies this number may considerably increase.

The fusion frequency of about 30 flashes per second is very fast compared to the contractions of invertebrate muscles, and is about equal to many vertebrate skeletal muscles (see Prosser et al., '50, p. 584). Among invertebrates, only certain insect muscles require a high frequency for fusion of contractions. This fast frequency of light response compared to most invertebrate muscle is probably connected with the fact that no movement of mass is involved as must inevitably take place in contractile processes. The frequency in the case of Mnemiopsis is actually limited by the rise time of a single flash.

In nearly every aspect, the light responses in a small meridional seection of Mnemiopsis tissue are analogous to those of muscle cells elicited by direct stimulation. This similarity leads to a suspicion that the photogenic process may be controlled by some sort of muscular activity at or near the site of photogenesis. Dahlgren ('16), in his study of Pleurobranchia pileus sections, believed that the luminous cells were confined within the meridional canal, forming a distinct layer covering the sex cells. Hertwig (1880) also reproduced a cross section of a meridional canal very similar to Dahlgren's section, but without identifying the photogenic cells. In neither case was there mention of muscle fibers within the canal or near light-producing cells. Further histological work is needed for positive identification of photogenic cells and nervous elements connected with them, but it seems most unlikely that any muscle fibers are associated with the luminous responses of ctenophores. The analogy observed between muscle contraction and light production is to be expected, since both the muscle fibers and the photogenic cells are the final effectors in a reflex arc of two different but comparable irritable systems.

The logarithmic nature of the decay phase of a Mnemiopsis flash suggests that the luminescent process, starting from maximal light and hence maximal luciferin concentration, proceeds as a simple first order reaction. The $\mu$ value for this decay process is 12,000 calories, of the same order of magnitude as most of the chemical reactions involving enzyme systems.

The phenomenon associated with the potential changes recorded along the meridional canal region are uncertain at the present time. Electric potentials might result from the metachronic movement of ciliary combs, the contraction of muscle fibers, conduction of nerve impulses or the luminescent response itself. Since no action potentials are observed in the oral lobe region where there is an abundance of muscle fibers, it seems unlikely that the electrical variations are muscle potentials. No one has observed potentials accompanying ciliary waves or paddle plate waves. The fact that in a great majority of cases, the electrical potentials exactly coincide with the luminescence suggests that they represent either luminor nerve potentials or luminous cell potentials.

Potential differences between the luminous organ region and other parts of the body when the organ is luminescing have been observed by Hasama ('39–'44) in many luminous forms: (firefly, '39, '42a, b, c, '44; Chaetopterus, '41a; squid, '41b; nudibranchs, '43). His monophasic records show negativity in the luminous regions, and the potential change usually started a little before and ended shortly after the luminescence. In the firefly the potential change showed the same rhythmic changes as the flash pulsation. Since the light response faithfully follows the time course of the potential change, it would seem that he had measured the potential changes connected with luminous cell function.

## SUMMARY

1. The bioluminescent system of *Mnemiopsis leidyi* gives flash responses quite analogous to contraction responses in muscle fibers when directly stimulated, and is well adapted for quantitative physiological analysis.

2. The light from a small piece of meridional canal containing 4 or less paddle plates placed in a specially designed chamber and stimulated by an electronic stimulator can be detected by a photomultiplier tube, amplified, and recorded photographically from a dual-beam cathode ray oscillograph.

3. Such a preparation gives a single flash after each stimulation. The intensity of the flash increases gradually as the voltage or the duration of the stimulating pulse is increased.

A decrease of stimulus intensity will diminish the flash intensity.

4. The time course of a single flash has been analyzed at 5 different temperatures between 6° and 35°. The latent period varies widely, from 4.7 to 37.5 msec. at room temperature, depending on the conditions of stimulation. Increased voltage decreases and increased duration increases the latent period. At 21.0–23.5°C. the half rise time averages 34.7, the full rise time 59.9, the half decay time 47.8, the nine-tenths decay time 114.3, and the full decay time 230 msec.

5. Raising the temperature shortens the time course of each flash, especially the decay phase. Lowering the temperature has the opposite effect.

6. The decay phase of a flash is logarithmic within the temperature range of 12° to 35°. The Arrhenius temperature characteristic, $\mu$, has been determined within the above range of temperature by plotting the logarithm of the decay rate versus the reciprocal of the absolute temperature, giving a value of about 12,000 calories.

7. Two or more subthreshold stimuli will produce a light response.

8. Two or more stimuli given in a shorter time than the duration of single flash result in light responses analogous to summation of muscle twitches. At a stimulation frequency of about 30 per second the light responses will fuse.

9. Phenomena resembling treppe of muscle or neuromuscular facilitation have also been observed in luminous responses when a series of stimuli of the same intensity are given.

10. Sustained light production comparable to complete and incomplete tetanus of muscle also occurs at a higher frequency of stimulation.

11. Fatigue in this luminous system appears rapidly. The time of appearance of fatigue is affected by both the frequency and the strength of stimulation.

12. The response in a whole animal or an isolated whole meridional canal to a single stimulus is irregularly repetitive in nature, but is never sustained for any length of time.

13. The conduction speed of the excitation of luminescence in an isolated whole canal has been measured by means of two photomultiplier tubes, responding to light at two points on the canal, and found to average 13.7 cm per second.

14. Using an A.C. amplifier with Ag-AgCl electrodes placed on a canal to record potential changes, and a photomultiplier detector for light responses, action potentials synchronous with the light flashes have been recorded. The action potentials appear as rapid bursts, each of the order of 30 msec. duration, rather than slow potential changes exactly following the light response. Similar and other types of action potentials when no luminous response is involved have also been observed, and it is concluded that they accompany nerve impulses rather than photogenic processes.

## ACKNOWLEDGMENTS

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# SOME RELATIONSHIPS BETWEEN ACTION POTENTIAL, OXYGEN CONSUMPTION AND COENZYME CONTENT IN DEGENERATING PERIPHERAL AXONS

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NINE FIGURES

## INTRODUCTION

In order to gain additional insight into the relationship between metabolism and function in nervous tissue, studies were undertaken on the changes in metabolic state which accompany loss of function during degeneration, *in situ* and excised, of peripheral axons of the frog.

The maximal amplitude of the compound action potential of the A-fibers was used as a measure of the number of axons capable of conducting impulses. Respiration of the resting nerve and the activity respiration (that is, the extra oxygen consumption associated with conduction of impulses) were used as measures of the metabolic state of the nerves. However, a study of the oxygen consumption gives very limited information regarding changes in the intermediary metabolism of the nerve. Accordingly, the changes in certain chemical components known to be of central importance in the intermediary metabolism of most tissues were also studied. The components were the pyridine nucleotide coenzymes and adenosine triphosphate (ATP).

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The results to be described suggest that the loss of excitability during degeneration *in situ* may be ascribed to metabolic defects fundamentally different from those causing failure of excised nerves.

## METHODS

*1. Degeneration of excised axons.* Both sciatic nerves of the grass frog, *Rana pipiens,* were excised. The nerves were kept at 20°C. in a non-nutrient solution of 111 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 4.8 mM $NaHCO_3$ and 100 μg/ml chloromycetin under an atmosphere of 20% $O_2$, 78% $N_2$ and 2% $CO_2$ (pH 7.0).

After a two-hour equilibration with this solution, one nerve which served as the control was extracted and its content of pyridine nucleotides and ATP determined. The second nerve was allowed to degenerate.[4] The amplitude of the compound action potential and the activity respiration of the A-fibers, as well as the resting respiration of the whole nerve were measured after equilibration in the non-nutrient solution. The same three measurements were made on sucessive days thereafter. The results of the latter measurements are reported as percentages of the corresponding measurements obtained on the day of excision. Finally, the nerve was extracted and analyzed for pyridine nucleotide and ATP content. The concentrations of these substances are reported as percentages of the concentrations of the corresponding substances in the control nerve.

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[4] To minimize the bacterial contamination which occurred in these solutions during nerve degeneration, several antibiotics were tested. Chloromycetin (chloramphenicol, Parke, Davis and Company) in a concentration of 100 $\mu$g/ml was finally selected for use in these experiments since it was found to reduce the rate of bacterial growth and also to prolong the time required for a 50% reduction of the action potential (from an average value of about two days without chloromycetin to three days with chloromycetin). In order further to minimize bacterial contamination, the solutions bathing the nerve were changed every 12 hours. In almost all degeneration experiments with excised nerves, bacterial counts were made on serial dilutions of the bathing medium after 48-hour incubation at 23° on nutrient-agar plates. In spite of the precautions taken, there was, nevertheless, significant bacterial contamination in the long term experiments. The following are representative values to indicate the order of magnitude of the bacterial count per milliliter after various periods of nerve degeneration under the conditions described: 1 day, $10^3$; 2 days, $10^4$; 3 days, $10^5$; 4 days, $10^6$; 5 days, $10^7$. Without chloromycetin, the counts were approximately 100 times as high.

*2. Degeneration in situ.* The frogs were anaesthetized by immersion in a solution containing 4–5 mM chloretone. One sciatic nerve was severed near its site of emergence from the spinal cord (i.e. the 7th, 8th, and 9th spinal nerves were cut). The blood supply to the nerve was left intact. Crystalline penicillin-G (Parke, Davis and Company) was applied to the cut dorsal surface and the skin sewed together with nylon thread. The animals showed rapid recovery from the operations. After varying periods of time (three to 22 days post-operative), the frogs were sacrificed and both sciatic nerves were removed. The solution used to bathe these nerves after excision was the same as described for the studies on degeneration of excised nerves except that chloromycetin was omitted. Following equilibration with the standard Ringer's solution, the action potential, resting and activity respiration of each nerve were measured. Upon completion of these measurements the nerves were extracted for the determination of pyridine nucleotide and ATP content. The value of each of the 5 measurements on the severed nerve is reported as the percentage of the value of the corresponding measurement on the control nerve from each animal.

*3. Action potential, resting respiration and activity respiration.* These properties were measured in an oxygen cathode flow respirometer provided with electrodes for stimulating the nerve and recording its action potential. This equipment has been described in detail elsewhere (Carlson, Brink and Bronk, '50). After the resting rate of respiration was established, the compound action potential of the A-fibers was measured by means of recording electrodes, an amplifier, and an oscilloscope. Following this, the A-fibers were stimulated at a frequency of 100/second for activity respiration measurements. These measurements were carried out in either of two ways (Brink, Bronk, Carlson and Connelly, '52): (A) by determination of the total quantity of extra oxygen consumed as a result of a 5-minute period of stimulation; (B) by measurement of the increment in steady-state rate of oxygen consumption resulting from continuous stimulation. The former method was used unless otherwise noted. The action potential was monitored throughout the stimulation period associated with measurement of the activity respiration.

TABLE 1

*Average values for resting and activity respiraton of control nerves (measured on day of excision)*

The number of cases is indicated in parentheses and the variability is indicated by the average deviation from the mean

| SOURCE OF NERVE | ADDITIONS | RESTING RESPIRATION | ACTIVITY RESPIRATION | |
|---|---|---|---|---|
| | | | Method A | Method B |
| | | *µmols $O_2$/g/hour* | *µµmols $O_2$/g/impulse* | *µmols $O_2$/g/hour* |
| Normal frog | None | 2.28 ± 0.49 (4) | 6.91 ± 0.47 (4) | 1.10 ± 0.24 [1] |
| Normal frog | Chloromycetin $\frac{100\ \mu g}{ml}$ | 1.92 ± 0.47 (12) | 7.81 ± 1.23 (7) | 1.11 ± 0.16 (4) |
| Operated frogs [2] | None | 1.94 ± 0.36 (12) | 7.48 ± 0.98 (10) | |

[1] Values reported by Carlson and Brink, '51.

[2] Control nerves excised 3–22 days after section of opposite nerve.

As mentioned above, the values for amplitude of action potential, resting and activity respiration in degenerating nerve are reported in this paper in terms of percentages of corresponding control values. The average absolute values found for respiration of control nerves are shown in table 1. The values found for nerves from normal frogs were in good agreement with those reported in the literature (Carlson, '48; Gerard, '32). Furthermore, the average values found for control nerves excised three to 22 days after section of the opposite nerve were not significantly different from normal, nor was there any evidence indicating a change in these control values in the long term *in situ* degeneration experiments. It can also be seen that chloromycetin had no significant effect on the resting or activity respiration of freshly excised nerves. There was no noticeable effect of chloromycetin on the action potential of the freshly excised nerves, nor was the action potential of the control nerves in the experiments on degeneration *in situ* significantly different from normal. The maximum amplitude of the action potential in the control nerves was about 4 mv under the conditions of recording from the nerve in the capillary of the respirometer.

4. *Pyridine nucleotide content.* The analytical method developed for use in the present experiments provides a true measure of the total pyridine nucleotide coenzyme content. Since triphosphopyridine nucleotide (TPN) occurs in much lower concentration than diphosphopyridine nucleotide (DPN) in various tissues (Schlenk, '42), including mammalian brain (McIlwain, '52), it seems likely that the pyridine nucleotide values reported in this paper primarily represent DPN. Nevertheless, the values are reported as pyridine nucleotide coenzyme content rather than as DPN content.

The following assay method was developed during these investigations for determining both the oxidized and reduced forms of DPN and TPN in the same sample of tissue: After weighing on aluminum foil, the nerve (about 25 mg) was wrapped loosely in the foil to facilitate handling and was dropped into a tube (13 × 100 mm) containing 3.0 ml of 0.1 M "Tris"[5] buffer (pH 8.2) which had been preheated to 100° in a boiling water bath. After 15 seconds, a time selected to minimize thermal[6] and enzymatic destruction of the pyridine nucleotides, the tube was chilled in ice. The contents were then homogenized by means of a glass pestle which had been ground to fit the lower part of the tube. Three milliliters of chloroform were added to the homogenate and the tube was stoppered with a cork and shaken for three minutes.[7] Finally the mixture was centrifuged at 2000 × g for 20 minutes and the clear supernatant was removed by means of a syringe and analyzed.

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[5] Tris (hydroxymethyl) aminomethane.

[6] A study was made of the effect of boiling both the oxidized and reduced forms of DPN at various pH's. It was found that at pH 8.2 (the pH used for boiling the nerves), there is less than 10% destruction of either form upon one minute boiling, so that the 15-second boiling time used for extraction should have produced negligible destruction.

The analytical procedure used is based on the fact (Robinson, Levitas, Rosen and Perlzweig, '47; Carpenter and Kodicek, '50) that compounds, in which oxidized (but not reduced) nicotinamide has a group substituted on the ring nitrogen, will condense with ketones to form a fluorescent product of unknown nature. (Such compounds will henceforth be referred to as "fluorogenic".) Since compounds in the extracts other than the pyridine nucleotides might give rise to fluorescence, appropriate enzymes were used in order to insure the specificity of the analytical procedure (Kaplan, Colowick and Barnes, '51).

Three 0.6 ml aliquots from each extract were placed in fluorometer tubes (Coleman No. 12-190) designated A, B, and C. The $PN^+$ [8] in tube C was split at the nicotinamide$^+$-ribose linkage by the addition of a hydrolytic enzyme from *Neurospora crassa.* This enzyme (DPNase) reacts only with $DPN^+$ and $TPN^+$ and not with DPNH nor with TPNH, nor with any other known substituted nicotinamide compound (Kaplan, Colowick and Nason, '51). Thus, by subtracting the fluorescence intensity of tube C from that of tube A, one obtains that due to the $PN^+$. The reduced pyridine nucleotide in tube B was oxidized (and thus converted to the fluorogenic form) by the addition of sodium pyruvate and lactic dehydrogenase from rabbit muscle (Kornberg and Pricer, '51). Thus, by subtracting the fluorescence intensity of tube A from that of tube B, one obtains the fluorescence due to the reduced pyridine nucleotides.

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[7] This step, based on the method of Sevag (Sevag, Lackman and Smolens, '38) was found to be necessary in order to obtain clear supernatants following centrifugation. The supernatants so obtained were protein-free as judged by testing for turbidity on addition of trichloroacetic acid. The solutions were also essentially free of enzymes acting on the pyridine nucleotides, as shown by the stability of added DPN during incubation with such extracts.

[8] The notation "$PN^+$" will be used, henceforth, to refer to the oxidized pyridine nucleotide coenzymes (the sum of $DPN^+$ and $TPN^+$).

A typical protocol for the pyridine nucleotide analysis was as follows:

| TUBE A | TUBE B | TUBE C |
|---|---|---|
| 0.6 ml unknown | 0.6 ml unknown | 0.6 ml unknown |
| | 0.1 ml Na pyruvate (M/60) | 0.1 ml DPNase[9] |
| | 0.1 ml lactic dehydrogenase[9] | |
| | 0.1 ml M/20 $KH_2PO_4$[9] | |
| Incubate for 2 hours at 37°C. | | |
| 0.1 ml M/20 $KH_2PO_4$ | | 0.1 ml M/20 $KH_2PO_4$ |
| 0.2 ml $H_2O$ | | 0.1 ml $H_2O$ |

To each tube add 0.2 ml of a 1:500 dilution of M/10 $MnCl_2$ in methyl ethyl ketone, 0.6 ml of 3.5 N NaOH, wait 5 minutes and then add 6.5 ml of 0.4 N HCl. Boil 5 minutes, cool and measure fluorescence with B-1, PC-1 filters in a Coleman Model 12B Electronic Photofluorometer.

The intensities of fluorescence were compared with those of standard solutions of $DPN^+$ and DPNH. The concentrations in the standard solutions were established by measurements of light absorption at 340 mμ before and after treatment with appropriate oxidizing or reducing enzyme systems (Schlenk, '42). In two experiments in which 0.2 μg internal $DPN^+$ standards were added to the nerve extracts the recoveries, by comparison with the external standard regularly used, were 105 and 110%.

The reproducibility of the method is shown in table 2. It can be seen that there is good agreement between paired nerves. The fraction of pyridine nucleotides in the oxidized form is in agreement with that reported for brain tissue, although higher than that reported for liver (Kohn, Klein and Dann, '39). Accordingly, the analytical method was further checked using 20 mg samples of rat liver. These results, also shown in table 2, are in good agreement with published values (Kohn, Klein and Dann, '39).

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[9] Appropriate concentrations of lactic dehydrogenase and DPNase, as determined by preliminary experiments, were used to insure that the respective reactions went to completion. In the case of DPNase, it is known that $DPN^+$ and $TPN^+$ are hydrolyzed at essentially equal rates (Kaplan, Colowick and Nason, '51). However, in the case of lactic dehydrogenase, it was found that TPNH was oxidized only 1/1500 as fast as DPNH. Therefore, it was necessary to use relatively large amounts of this enzyme to insure complete oxidation of both reduced nucleotides. The purpose of the addition of $KH_2PO_4$ was to change the pH from 8.2 to 6.8 since the rate of enzymatic oxidation of TPNH was found to be increased 10-fold by this pH change. Appropriate blank samples were prepared to correct for the small amount of fluorescence contributed by the two enzyme preparations.

$DPN^+$ and $TPN^+$ are stable to acid but destroyed by alkali, whereas DPNH and TPNH are stable to alkali but destroyed by acid. Therefore, acid and alkali extracts of tissues may be used for the individual determination of the oxidized and reduced forms of the coenzymes, respectively (Schlenk, '42).

TABLE 2

*Pyridine nucleotide coenzyme content of peripheral nerve* $\left(\frac{\mu mols}{gr.} \times 10^3\right)$

(L and R represent left and right nerves respectively)

| NERVE NO. | OXIDIZED | REDUCED | TOTAL | OXIDIZED RATIO (TOTAL) |
|---|---|---|---|---|
| 51 L | 96.5 | 17.3 | 113.8 | .85 |
| 51 R | 99.3 | 20.5 | 119.8 | .83 |
| 52 L | 89.2 | 26.0 | 115.2 | .77 |
| 52 R | 88.1 | 21.8 | 109.3 | .81 |
| Rat liver | 492.0 | 442.0 | 934.0 | .53 |

When different nerves were extracted by homogenizing with trichloroacetic acid or by boiling with alkali, the values found for the oxidized or reduced nucleotides respectively, were in agreement with those found by the extraction procedure developed for these studies. Acid and alkaline extractions were not feasible for use in the present experiments, since two samples of a single nerve would have been required to determine the relative amounts of oxidized and reduced forms. This would have been disadvantageous both because of the increased complexity of the procedure and more important, because it would have required the use of different tissue samples and could have been subject to inherent variability in composition which might have existed between different portions of the same tissue. With respect to the latter point, it has been established, using the pH 8.2 boiling procedure on freshly excised nerves, that no proximal-distal-gradient in pyridine nucleotide concentration is detectable and that the coenzyme is largely in the oxidized form throughout the length of the nerve. Corresponding measurements have not been made on degenerating nerve, but would be of interest.

5. *ATP content.*[10] The extracts prepared as described in the preceding section were also suitable for the ATP analyses. The amount of ATP in the supernatant was determined utilizing an enzyme system (luciferin-luciferase) extracted from the lantern of the firefly, *Photinus pyralis* (McElroy, '51). It has been shown (McElroy and Strehler, '49) that, under appropriate conditions, this enzyme system will produce a flash of light proportional to the amount of ATP added. Such conditions were established for these experiments. The method has been shown to be specific for ATP, under appropriate conditions, no detectable light being produced by ADP or inorganic polyphosphates (McElroy, '51). With the particular enzyme system used here, it was found that traces of light were produced by inorganic pyrophosphate and triphosphate but that the amounts of these substances required to produce a given response was about 20,000 times that required with ATP. The system used for these analyses consisted of 2.2 ml of 0.05 M glycine (pH 8.2), .13 ml of 0.10 M $MgSO_4$ and 0.1 ml of the enzyme system. One-tenth milliliter of either the supernatant from the nerves or standard solutions of ATP (Schwartz) were added to this system. The flash of light thus obtained was detected with a photomultiplier and recorded with an Esterline-Angus Model AW Graphic Meter (Hastings, McElroy and Coulombre, '53). It was found that the amount of chloroform present in 0.1 ml of the nerve extracts caused a 25% inhibition of the light response to ATP, but did not interfere with the linearity of the response with different amounts of ATP. Accordingly,

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[10] We are indebted to Professor William D. McElroy and Mrs. Jane Coulombre for making possible the determinations of adenosine triphosphate reported in this paper.

the standard solutions of ATP in 0.1 M Tris buffer (pH 8.2) were saturated with chloroform. The concentration of ATP in the standard solutions was estimated on the basis of the acid-labile phosphate reported by the supplier.

The reproducibility of this method is shown in table 3. It can be seen that there is good agreement between paired nerves. These analyses were carried out using late spring frogs. The values obtained are somewhat higher than were found for early spring frogs. The latter value (about 1.1 micromols ATP per gram) is in fair agreement with that reported by Gerard (8 mg % ATP phosphorus, corresponding to 0.9 micromols ATP per gram) for fall frogs (Gerard, '32).

TABLE 3

*ATP content of peripheral nerve*

| NERVE NO. | μMOLS<br>gr. | NERVE NO. | μMOLS<br>gr. |
|---|---|---|---|
| 115 L | 1.80 | 119 L | 1.87 |
| 115 R | 1.73 | 119 R | 2.01 |
| 116 L | 1.87 | 120 L | 1.87 |
| 116 R | 1.87 | 120 R | 1.77 |

Gerard also noted a seasonal variation in acid-soluble phosphorus content, which was about 50% higher in winter frogs, a finding supporting the view that the variations observed here were due to seasonal fluctuations.

6. *Weight of nerves.* It was found in the present investigations that nerves increase in weight (about 20% in three weeks) during degeneration *in situ,* presumably due to an increased hydration. [This observation agrees with others reported in the literature (Gerard, '32; Muralt and Schulthess, '44).] Accordingly, part of the decrease in the concentrations of the pyridine nucleotides and ATP observed during *in situ* degeneration was due to this increase in weight. A weight increase was also observed during degeneration of excised nerves. In the latter case, however, this factor could be neglected in the calculations by using the values of the weights as determined immediately following excision.

For both types of degeneration, the respirations of the experimental and control nerves have been compared by using the values for the oxygen consumed by the 22 mm length of nerve in the capillary of the respirometer rather than by using the values for $Q_{O_2}$. This procedure eliminated not only the problem of increasing weights cited above but also the errors inherent in weighing the approximately 7 mg of nerve in the capillary.

## RESULTS

*1. Action potential and respiration measurements.* The temporal changes in action potential and activity respiration of 5 nerves, following excision, are presented in table 4, together with the average values for each of these quantities. The values are expressed as percentages of the values of

TABLE 4

*Action potential and activity respiration during degeneration of excised nerves expressed as percentage of values on day of excision*

| NERVE | | TIME POST-OPERATIVE (IN DAYS) | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| No. 34 [1] | Action potential | 103.2 | 95.3 | 82.8 | 39.1 |
| | Activity respiration | 100.0 [2] | 67.3 | 30.9 | 0 |
| No. 36 [1] | Action potential | 105.5 | ... | 46.8 | 0 |
| | Activity respiration | 89.7 | ... | 7.4 | 0 |
| No. 53 [1] | Action potential | 98.7 | 89.6 | 26.0 | ... |
| | Activity respiration | 80.0 | 32.0 | ... | ... |
| No. 57 | Action potential | 94.3 | 99.4 | 62.8 | ... |
| | Activity respiration | 125.0 | 46.8 | ... | ... |
| No. 58 | Action potential | 102.0 | 102.4 | 64.2 | ... |
| | Activity respiration | 98.4 | 43.2 | ... | ... |
| Average | | | | | |
| | Action potential | 100.7 | 96.7 | 56.5 | 19.6 |
| | Activity respiration | 98.6 | 47.3 | 19.2 | 0 |

[1] Respiration obtained by steady-state procedure (see section 3 B in Methods).

[2] The activity respiration of Nerve No. 34 was not measured on the day of excision. Therefore, the value obtained one day post-operative is taken as 100%. This procedure was considered acceptable since the average value of the activity respiration of the other 4 nerves showed negligible change during the first day post-operative.

the respective parameters on the day of excision. In addition the average values are presented graphically in figure 1. It can be seen that the activity respiration decreases more rapidly than the action potential. Thus, at a time when the activity respiration has fallen to 50%, the action potential is more than 90% of its initial value. These observations are a repetition, under conditions of constant temperature, of the first experimental study of this phenomenon (Brink, Bronk, Carlson and Connelly, '52).

The percentage of action potential remaining, as a function of time post-operative, is shown in figure 2 for nerves degenerating *in situ*. For comparison, the action potential-time curve for excised degenerating nerves (fig. 1) is also shown in figure 2. The activity respiration follows a time course of decrease very similar to that of the action potential during *in situ* degeneration. This is brought out strikingly in figure 3 where, for both types of degeneration, the percentage of the activity respiration remaining is plotted as a function of the percentage of the action potential remaining. A straight line with a slope of 1 fits quite well the data for degeneration of nerves *in situ*. Thus, for severed nerves *in situ*, the increased respiration associated with impulse conduction is maintained intact as long as the action potential itself, whereas in excised nerves the increased respiration disappears prior to the failure of the action potential. The possible significance of this difference in the two types of degeneration will be discussed later.

The failure of the activity respiration prior to that of the action potential during degeneration of excised nerves has been studied under a variety of conditions. Thus, the temperature (in the range of 5° to 20°C.) and the pH (in the range of 6.4 to 7.0) of the solution in which the nerves were stored, the use or omission of chloromycetin, and the period of electrical stimulation used in testing the nerves did not alter the relative rates at which the values of these two parameters decreased even under conditions in which the absolute values of these rates were greatly changed. The cumulated data, if plotted in figure 3, would fall very closely along the curve for degeneration of excised nerves.

An increase in the resting respiration during *in situ* degeneration has been measured (table 5). The data on resting

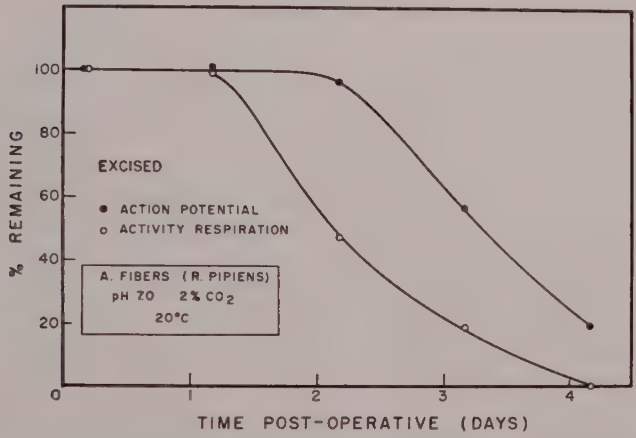

Fig. 1 Time course of changes in action potential and activity respiration during degeneration of excised nerves. Average values of measurements on 5 nerves.

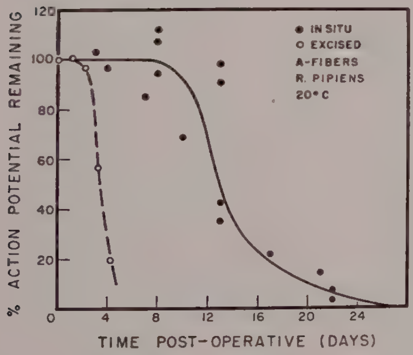

Fig. 2 Time course of change in action potential during degeneration *in situ*. Each solid circle represents data from one frog. The action potential curve for degeneration of excised nerves (fig. 1) is included for comparison.

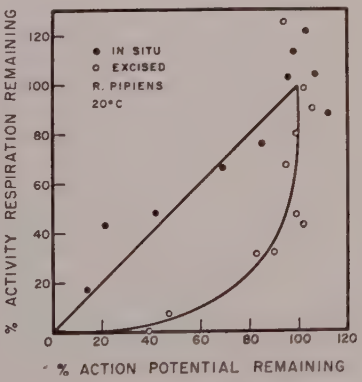

Fig. 3 Relationship between activity respiration and action potential during degeneration. Each solid circle represents data from one frog. The set of open circles represent the data from 5 nerves, as tabulated in table 4.

respiration for excised degenerating nerves are not presented, since it was not possible to estimate to what extent bacterial contamination was responsible for the large increases sometimes observed (cf. Brink, Bronk, Carlson and Connelly, '52).

*2. Coenzyme studies.* The data on the time course of disappearance of $PN^+$ during degeneration are presented in figure 4. In excised nerves, the $PN^+$ falls nearly to zero in about 5 days after excision. *In situ,* on the other hand, one can observe a much slower loss and an apparently asymptotic approach to a level at which about 40% of the $PN^+$ remains. This suggests that this 40% fraction is in the Schwann cells and connective tissue of the nerve trunk, since it is known that these tissues do not degenerate *in situ* after sectioning of the nerve (Young, '42).

TABLE 5

*Resting repiration during in situ degeneration expressed as percentage of respiration of control nerve*

| NERVE | TIME POST-OPERATIVE | RESPIRATION |
|---|---|---|
| | *in days* | |
| No. 92 | 3 | 65.3 |
| No. 73 | 4 | 104.8 |
| No. 68 | 7 | 97.8 |
| No. 79 | 8 | 95.3 |
| No. 94 | 8 | 118.4 |
| No. 70 | 10 | 147.6 |
| No. 77 | 13 | 144.6 |
| No. 91 | 13 | 136.1 |
| No. 80 | 17 | 218.0 |
| No. 62 | 21 | 166.6 |
| No. 95 | 22 | 172.0 |
| No. 96 | 22 | 130.7 |

The probable nature of the $PN^+$ destruction is suggested by the present experiments. The disappearance is not due to conversion to reduced forms (DPNH and TPNH). Furthermore, it is probably not due to a splitting of the $PN^+$ at the prophosphate linkage to form nicotinamide$^+$-ribose-phosphate. Although two enzymes are known to split $DPN^+$ at that linkage, the existence of such enzymes in brain or nerve tissue has not been described. Furthermore, if such a splitting had occurred in the present experiments, analysis of the extracts of aged nerves would have been expected to show the appearance of a DPNase-resistant fluorogenic compound, namely nicotinamide⁺-ribose-phosphate. Experimentally no evidence was found for the appearance of such a compound in the nerves during degeneration, the loss of $PN^+$ observed being in all cases accounted for by a decrease in fluorogenic substances. This result suggests that the $PN^+$ which disappeared had been split at the nicotinamide⁺-ribose linkage by nerve DPNase. This observation is in agreement with the high activity of this type of DPNase found in brain homogenates (McIlwain, '52). The possibilities exist, however, either that $PN^+$ was split at the pyrophosphate linkage and that the

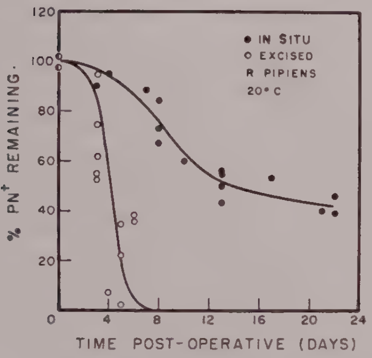

Fig. 4 Time course of change in concentration of $PN^+$ (the sum of the oxidized pyridine nucleotide coenzymes, $DPN^+$ and $TPN^+$) during degeneration. In most cases it was established that the reduced form constituted a small fraction (about 15%) of the total pyridine nucleotide. (In the remaining cases only the oxidized form was measured.) Each circle represents data from one frog.

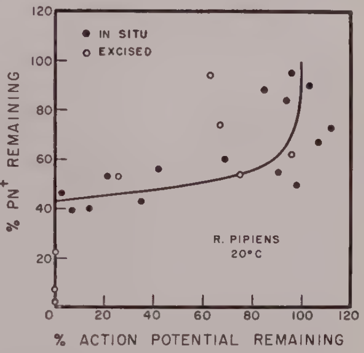

Fig. 5 Relationship between $PN^+$ (the sum of $DPN^+$ and $TPN^+$) and action potential during degeneration. The curve has been drawn to fit the *in situ* data. Each circle represents data from one frog.

nicotinamide$^+$-ribose-phosphate thus formed was able to diffuse out of the nerve, or even that DPN$^+$ and TPN$^+$ were able to diffuse out of the nerve.

The relationship between the PN$^+$ content and action potential of the nerves is shown in figure 5. The curve has been drawn to fit the data for nerves *in situ.* It can be seen that the PN$^+$ falls more rapidly than the action potential in the early stages of *in situ* degeneration and can reach values as low as 50% before there is an appreciable fall in action potential. In the later stages of *in situ* degeneration, the PN$^+$ falls to the 40% level mentioned previously. The data from the studies on excised nerves are also presented. No attempt has been made to fit the latter data with a curve. Obviously it is difficult to draw conclusions concerning the relative rates of fall of PN$^+$ and action potential in the excised nerves. However, it is clear that in this case, complete loss of action potential is accompanied by essentially complete loss of PN$^+$.

The data on ATP content during degeneration are presented in figure 6. The rate of disappearance of ATP from excised nerves was relatively rapid, a fall to 35% of the control value occurring in three days at which time the action potential was still 75% of normal. In striking contrast, it can be seen that the ATP falls at a very slow rate during *in situ* degeneration. Thus, at 22 days post-operative, a time when the action potential has fallen to a few per cent of its control value (see fig. 2), the ATP concentration is over 80% of that in the control nerve. Even this small change in concentration of ATP may be entirely due to the fact that the severed nerves had gained weight, presumably by absorbing water (see Methods). It is possible that there was no loss of ATP. The relationship between ATP and action potential is presented in figure 7. The curves there illustrate, more strikingly than does figure 6, the difference in the two types of degeneration. Thus, for *in situ* degeneration it is seen that the ATP level is still high at a time when the action potential has almost entirely disappeared. In excised nerve, on the other hand, the ATP has fallen to a low level prior to appreciable failure of the action potential.

In figure 8, the ATP remaining is plotted against the activity respiration remaining. One may observe that the

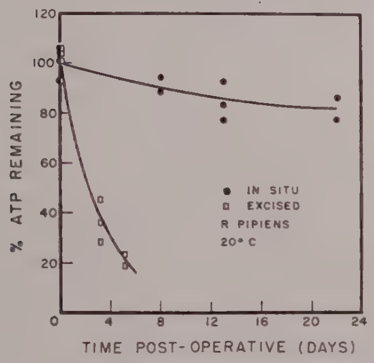

Fig. 6 Time course of change in concentration of ATP during degeneration. Each circle represents data from one frog.

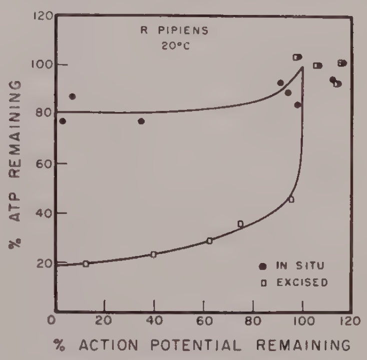

Fig. 7 Relationship between ATP and action potential during degeneration. Each circle represents data from one frog.

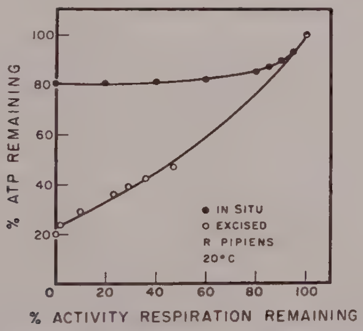

Fig. 8 Relationship between ATP and activity respiration during degeneration. This graph has been constructed from the curves drawn in figures 3 and 7 by eliminating the action potential as a variable.

values of these two parameters fall in a similar way during degeneration of excised nerves. In the case of *in situ* degeneration, of course, the relation between ATP content and activity respiration is the same as that already described between ATP content and action potential.

## DISCUSSION

The observed rate of loss of action potential of the A-fibers of nerves degenerating *in situ* is in agreement with that found by Gallego ('51) working with the same preparation. He reports that the action potential of the A-fibers starts falling on the 7th day and on the 15th day has reached 1/40 of its initial value. He states that "the daily decrease in the height of the conducted spike means that every day a number of fibers became inexcitable which up to that day had retained a great deal of functional ability" (as opposed to the idea that each of the fibers makes progressively smaller contributions to the action potential each day). Furthermore, according to Gallego, a "very advanced stage of Wallerian degeneration" is reached during this period. The loss of injury potential of peripheral nerves during degeneration *in situ* has been observed also (Koch, '25).

The histological picture of the degeneration of excised nerves is similar to that of nerves degenerating *in situ* (Cajal, '28; Nageotte, '32). The autolysis of the myelin and axis cylinders of excised nerves follows a pattern identical with, although more rapid than, that seen in Wallerian degeneration. In contrast, the Schwann cells, rather than proliferating as in Wallerian degeneration, die during the degeneration of the axons in excised nerves.

Perhaps the most striking result of the present investigation is the finding that during nerve degeneration *in situ*, the ATP content remains unaltered as the action potential falls to zero. This is in distinct contrast to the results of degeneration studies with excised nerves, in which case there is a marked decrease in ATP preceding the fall in action potential. The fact that excised nerves exhibit loss of excitability at a time when nerves degenerating *in situ* are not detectably altered suggests that the failure of the excised nerves is due to the absence from the bathing medium of certain substances normally supplied by the blood. These substances are apparently necessary in order to maintain the ATP concentration in nerve tissue and the *in situ* experiments suggest that as long as these substances are present, the ability to synthesize ATP remains intact. Thus while the failure of excised nerves is, perhaps, simply attributable to loss of the necessary energy supplying compounds, the failure of nerves degenerating *in situ* is apparently due to the development over a longer period of time of a fundamentally different defect.

The studies on activity respiration may be considered as supporting this view as to the difference between the mechanisms of loss of excitability in the two types of degeneration. According to the view represented in figure 9, the energy of ATP is available for maintaining a concentration gradient of sodium ions between the inside and the environment of axons. (It may be emphasized that no part of this discussion is critically dependent upon the sodium ion hypothesis. Thus, for present purposes, one might equally well have placed emphasis on any other substance involved in impulse conduction.) The ATP is resynthesized by oxidative phosphorylation processes. In the resting nerve, steady-state conditions exist. An unknown fraction of the resting oxygen consumption presumably is used for extruding the sodium ions which enter the axons, owing to the permeability to sodium ions of the resting membrane. During conduction of impulses the inward flux of sodium ions increases, resulting in a compensatory increase in oxygen consumption.

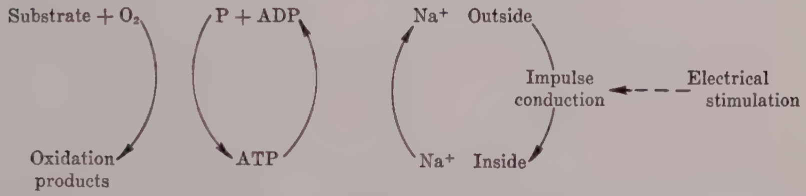

Fig. 9 A hypothetical scheme based on some concepts, currently being considered by nerve physiologists, inter-relating certain measurable properties of normal nervous tissue. The solid arrows represent energetic couplings rather than individual chemical reactions.

According to this view, the activity respiration may be regarded as a measure of the ability of the axons to resynthesize ATP after passage of an impulse. Considered in this way the finding that the failure of the activity respiration precedes that of the action potential in excised nerves would again implicate the system for the maintenance of ATP as the locus of the defect responsible for failure under such conditions. In contrast, during nerve degeneration *in situ,* the percentage of activity respiration remaining is equal to the percentage of active axons remaining (as measured by the action potential), suggesting that the activity respiration in the active axons is unchanged. According to the view discussed above, this would again indicate that the system for the maintenance of ATP was intact during *in situ* degeneration, at least up to the onset of inexcitability in each axon. Therefore, it would seem that a defect more intimately associated, than is the ATP system, with the conducting mechanism is responsible for the loss of the action potential during degeneration *in situ.*

The increase in resting respiration observed during *in situ* degeneration agrees with other observations suggesting increased metabolism of peripheral axons during degeneration. Thus, the activity of acid phosphatase (Bodian, '47), and the uptake of $P^{32}$ labelled inorganic phosphate are increased during Wallerian degeneration of the mammalian sciatic nerve. The increased phosphate uptake has been shown to be associated with acid-soluble phosphate (Bodian and Dziewiatkowski, '50), phospholipids (Bodian and Dziewiatkowski, '50; Samuels, Boyarsky, Gerard, Libet and Brust, '51), nucleic acid and phosphoprotein phosphates (Samuels, Boyarsky, Gerard, Libet and Brust, '51). It is possible that these observed increases in the rates of respiration and phosphorus metabolism are attributable to the increased number of Schwann cells occurring during Wallerian degeneration of the sciatic nerves. On the other hand, observations cited by Gerard ('32) indicate that following section of the white matter of the spinal cord, there is a marked increase in oxygen uptake, apparently directly related to an "increased unsaturation of fatty acids."

Two studies on the content of acid-soluble phosphate during degeneration *in situ* of the sciatic nerve are of interest in connection with the present observations on the maintenance of ATP under such conditions. Samuels et al. ('51) report no change in acid soluble phosphate, Bodian and Dziewiatkowski ('50) observe only a slight decrease during degeneration. It is, of course, possible that much of the ATP and other acid-soluble phosphates is located in the sheath cells. If this should be proven correct a considerable revision of present ideas about the metabolism of axons would be required.

The decrease in concentration of the pyridine nucleotides, approaching a level 40% of the initial value, during degeneration *in situ,* is very similar to the behavior of at least two other compounds. Thus, during Wallerian degeneration of the sciatic nerve of the guinea pig, both aneurin (Muralt and Wyss, '44) and "true" cholinesterase (Sawyer, '46)[11] content decrease to a level about 40% of their initial values. It seems quite possible that these 40% residues may be located in the Schwann cells. On the other hand, from 90 to 100% of the acetylcholine content is lost from that preparation following nerve section (Muralt and Schulthess, '44). The complete loss of pyridine nucleotides after extensive degeneration of excised nerves may be correlated with the death of the Schwann cells under these conditions.

The possibility has been experimentally excluded that the cell bodies maintain the integrity of peripheral fibers merely by sending impulses along them. Thus, stimulation of nerves either excised (Abrams and Gerard, '33) or *in situ* following section (Cook and Gerard, '31) hastens the failure of the action potential. Gerard, reviewing evidence that the spread of chemicals along axis cylinders occurs at much higher rates than would be attainable by simple diffusion, has suggested (Gerard, '32) that degeneration following nerve section is attributable to interference with the chemical transport of substances formed in the cell bodies. The recent studies of Weiss and Hiscoe ('48) provide convincing evidence for a continual flow of axoplasm distad from the cell bodies. Evidence of a 2.7 mm per day movement of phosphoprotein distad along the nerve (Samuels, Boyarsky, Gerard, Libet and Brust, '51) (a rate in agreement with that for fiber outgrowth) further supports this idea. Evidence of the passage of $P^{32}$ compounds along peripheral nerves has recently been reviewed (Dawson, '52). The results of the present experiments on degeneration *in situ* are compatible with the view that either the pyridine nucleotide coenzymes or precursors of these substances are supplied to the axons by the cell bodies. It is of interest in this connection that Hogeboom and Schneider ('52) have obtained evidence indicating that the synthesis of DPN in rat liver takes place exclusively in the cell nuclei, the DPN subsequently diffusing out to the cytoplasm. As mentioned above, the present studies suggest that ATP synthesis, on the other hand, can proceed in axons severed from their cell bodies.

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[11] The paper by Sawyer ('46) reconciles much of the earlier conflicting evidence regarding the loss of cholinesterase during degeneration.

The question of the possible functional significance of the DPNase reaction in nervous tissue has been raised by McIlwain ('52). More recently Woolley ('53) has also considered this problem. The latter author has suggested the possibility that the conversion of quaternary ammonium compounds, such as thiamine and diphosphopyridine nucleotide, to the corresponding tertiary amines, with the concomitant production of hydrogen ions, might be intimately involved in the propagation of nerve impulses. In this connection, the observation (Alivisatos and Denstedt, '51) that the DPNase of red blood cells is apparently concentrated in the cell membranes is suggestive. A study of the spatial distribution of DPNase in the giant axon of the squid would be of some value in assessing the idea of a direct functional role of DPNase in impulse conduction.

The data in this paper pertaining to the deterioration of excised nerve in non-nutrient media provide a basis for a systematic study of added substances that should, at best, make the excised nerve change as does a nerve degenerating *in situ* with intact blood supply. A nutrient solution suitable for tissue culture of Schwann sheath cells may be adequate for this purpose. Such an extended study is desirable then for the search for specific substances supplied to the axon by the cell body could be made, using excised axons in such a known environment.

## SUMMARY

1. Studies of degenerating peripheral axons have been undertaken in an attempt to obtain information correlating functional and metabolic properties of these axons. Degeneration has been studied both with excised nerves (kept in Ringer's solution) and *in situ* (in which the axons were severed from their cell bodies but left with intact blood supply). The variables measured in these studies were (a) the compound action potential of A-fibers, (b) the resting respiration, (c) the activity respiration of A-fibers, (d) the concentrations of both the reduced and oxidized forms of the pyridine nucleotide coenzymes and (e) the concentration of ATP.

2. Evidence is presented that the two types of degeneration are qualitatively different. The following results are given particular emphasis:

Firstly, *in situ* the activity respiration is maintained intact as long as the action potential itself, whereas in excised nerves the activity respiration disappears prior to the failure of the action potential.

Secondly, the ATP content of nerves degenerating *in situ* decreases only slightly, if at all, during the time in which the failure of the activity respiration and action potential occur. In contrast, the degeneration of excised nerves is accompanied by a rapid decrease in ATP content, this decrease actually preceding the failure of the action potential.

3. It is proposed that failure of excised nerves is associated with a defect in the system for the maintenance of ATP, whereas *in situ* failure is associated with loss of some factors more intimately connected than is the ATP system with impulse conduction.

4. Nerves degenerating *in situ* show a marked increase in their resting rates of oxygen consumption.

5. The pyridine nucleotide content of nerves degenerating *in situ* decreases prior to the failure of the action potential, approaching a final concentration about 40% of the initial value. The pyridine nucleotide content also decreases during degeneration of excised nerves approaching a final concentration of zero.

6. About 85% of the pyridine nucleotide coenzymes in peripheral nerve axons is in the oxidized form. The ratio of oxidized to reduced coenzyme is not altered significantly during degeneration. The destruction of the pyridine nucleotides is apparently due to a hydrolytic removal of the nicotinamide moiety of these coenzymes.

7. A method is described for the determination of both the reduced and the oxidized forms of the pyridine nucleotide coenzymes in a single tissue sample.

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# A COMPARATIVE STUDY OF THE OXYGEN DISSOCIATION OF MAMMALIAN HEMOGLOBIN [1, 2]

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TWO FIGURES

## INTRODUCTION

The voluminous literature of the comparative aspects of oxygen-hemoglobin equilibrium has received several complete reviews (Redfield, '33; Florkin, '34; Prosser, '50).

Krogh and Leitch ('19) reported that, in both fresh and salt water, species of fish which frequently encounter low oxygen tensions have hemoglobins with higher oxygen affinities than those of species which seldom or never encounter low oxygen tensions. Redfield, Coolidge and Hurd ('26) reported similar findings for several invertebrate species. These authors also pointed out that active species are likely to present hemoglobins with low oxygen affinities and high Bohr effects, while sluggish species are likely to possess hemoglobins with high oxygen affinities and low Bohr effects. Hall and McCutcheon ('38) revealed, for marine fish, data similar to those of Krogh and Leitch. Hall, Dill and Barron ('36) and Hall ('37) stated that mammalian and avian species indigenous to the Chilean Andes revealed hemoglobins with higher oxygen affinities than those of their sea level counterparts. The works cited above indicate that the oxygen affinities of specific hemoglobins are correlated with habits and habitats and bear little relevancy to phylogenetic relationships.

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[1] This work was done in the laboratories of the Department of Physiology and Pharmacology, Duke University Medical School, Durham, N. C.

[2] These findings were presented as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Schmidt-Nielsen and Gjönnes ('52) reported that a survey of existing literature indicated that, among mammalian species, those with greater body weights exhibit bloods with higher oxygen affinities than those of species with lesser body weights. These authors suggested that the latter correlation might be only secondary to the greater oxygen demands of the tissues of smaller mammals.

The species employed in many comparative studies of oxygen-hemoglobin affinities were members of different orders or classes and seldom belonged to the same family. The writer felt that a study of this type utilizing closely related species with similar habits and habitats might shed light on the correlations mentioned above. It was with this view in mind that this investigation was undertaken. It seemed that mammals offered the greatest similarity of habits and habitats, and they were chosen as subjects. The constancy of their body temperatures is of obvious advantage here. Since rodents were the most plentiful (in terms of number of species) of available animals, they were utilized for the major part of the work described below.

Instead of whole blood, carefully prepared dilute hemoglobin solutions were used for two reasons. First, results obtained from the former cannot easily be compared when the whole bloods of different species are involved. Second, the use of dilute hemoglobin solutions permits one to avoid pooling blood when the individuals are very small. Since dilute hemoglobin solutions under carefully controlled but rather arbitrary conditions were employed, the results reported here should be looked upon more as comparative chemistry than physiology.

## MATERIALS

The following animals were used in this experiment:

Cricetidae —

| | |
|---|---|
| 1. White-footed mouse * | Peromyscus leucopus |
| 2. Golden mouse * | Peromyscus nuttalli |
| 3. Harvest mouse * | Reithrodontomys humulus |

| | |
|---|---|
| 4. Pine mouse * | Pitymys pinetorum |
| 5. Meadow mouse * | Microtus pennsylvanicus |
| 6. Cotton rat * | Sigmodon hispidus |
| 7. Golden hamster | Mesocricetus auratus |
| Muridae — | |
| 8. House mouse * | Mus musculus |
| 9. White mouse | Mus musculus |
| 10. Norway rat * | Rattus norvegicus |
| 11. White rat | Rattus norvegicus |
| Sciuridae — | |
| 12. Gray squirrel * | Sciurus carolinensus |
| 13. Flying squirrel * | Glaucomys volans |
| Caviidae — | |
| 14. Guinea-pig | Cavia porcellus |
| Leporidae — | |
| 15. Domestic rabbit | Oryctolagus cuniculus |
| Soricidae — | |
| 16. Short-tailed shrew * | Blarina brevicauda |

Those animals with asterisks following their common names were collected by the author in Durham County, North Carolina, for the most part in the Duke University Forest. Others, not so designated, were common domesticated animals from the Duke University animal stocks. The white rats employed in this investigation were from the Osborne-Mendel Strain. The white mice were from a strain developed by the New York State Hygiene Department.

In addition to data for the animals listed above, the author has provided a dissociation curve determined from his own hemoglobin which should be considered as a standard for comparison of the other animals.

It should be noted that animals one through 14 are true rodents, while the rabbit and shrew are of the orders Lagomorpha and Insectivora respectively.

## METHODS

Blood for determinations of oxygen dissociation curves was obtained from the animals listed above by heart puncture.

Only 0.25 to 1.0 ml was required. Immediately upon withdrawal, the blood was laked in 14 ml of distilled water and was centrifuged for 20 minutes. The clear supernatant hemoglobin solution was then pipetted into a 100 ml graduated cylinder. Further dilution was effected with distilled water and 1/15 M phosphate buffer in such a manner that the final solution was one of hemoglobin in 1/30 M buffer of desired pH. This was the solution upon which the oxygen dissociation curves were established; it represented a dilution of one part whole blood in approximately 120 parts buffer solution. The cylinder and solution were kept in ice water until used. Five milliliter samples of the above solution were transferred to special tonometers and were equilibrated at different oxygen tensions in a constant temperature water bath at 37 ± .03°C.

The techniques of equilibration and analysis were essentially the same as those described by Hall ('34 and '35). The same apparatus developed by Hall was employed in this operation. It should suffice to mention that analysis involved visual comparison of the absorption spectrum of the equilibrated sample with that of a standard of known proportions of reduced and oxygenated hemoglobin.

Individual points established in this manner were plotted on special coordinate paper described by Brown and Hill ('22), and the curves presented here were extrapolated from these. Only in the case of the shrew were all the points on the dissociation curve obtained from one individual. For all the other species separate dissociation curves were established for each of at least two individuals, and in most cases three or 4 different ones were utilized.

Dissociation curves were established for each species at pH 6.80. Dissociation curves were also established for 6 species at pH 7.20. From these two curves an appreciation of the Bohr effect can be gained.

## RESULTS AND DISCUSSION

Table 1 summarizes the principal findings for each species. The species are arranged in the decreasing order of the affinity of their hemoglobins for oxygen as judged by the $pO_2$ necessary for half saturation. It is easily seen that there may be variation in oxygen-hemoglobin affinity among closely related species, even when these species also have similar habits and habitats. However, it should be pointed out that all the rats and mice examined fell within the rather narrow range of 32 to 37 mm Hg $pO_2$ necessary for half saturation of their hemoglobin with oxygen.

TABLE 1

*Comparative data for all mammals studied in this investigation*

| SUBJECT | $pO_2$ mm Hg; Hb = $HbO_2$ | | R = $\frac{\log (\Delta\, pO_2;\ Hb = HbO_2)^2}{2\, \Delta\, pH}$ | BODY WT. |
|---|---|---|---|---|
| | pH 6.80 | pH 7.20 | | *gm* |
| Human | 21.7 | | | 70,000 |
| Hamster | 22.0 | 13.7 | 2.29 | 88 |
| Guinea-pig | 24.0 | 14.0 | 2.50 | 514 |
| Rabbit | 24.8 | | | 3,370 |
| Gray squirrel | 26.2 | | | 400–450 |
| Flying squirrel | 30.1 | | | 45–70 |
| Harvest mouse | 32.2 | | | 10–15 |
| White rat | 32.4 | 19.7 | 2.76 | 387 |
| Short-tailed shrew | 32.8 | | | 15–27 |
| Cotton rat | 33.0 | 18.6 | 2.89 | 10–120 |
| White-footed mouse | 33.2 | 20.0 | 2.80 | 15–25 |
| Meadow mouse | 33.8 | | | 39 |
| Pine mouse | 34.0 | | | 25–35 |
| White mouse | 34.2 | | | 29 |
| House mouse | 34.2 | | | 14–24 |
| Norway rat | 35.5 | 20.3 | 2.95 | 300–540 |
| Golden mouse | 36.7 | | | 20–26 |

The ratio R in table 1 is provided as an index to the degree of the Bohr effect of the specific hemoglobins. It seems evident that, in the species examined, decreasing hemoglobin-oxygen affinity is accompanied by increasing Bohr effect. Such a relationship might be advantageous when metabolic demands require a high $O_2$ unloading tension at the tissue level. A similar relationship between oxygen affinity and Bohr effect appears in data published by Hall and McCutcheon

('38) for teleosts and in data for certain molluscs, by Redfield et al. ('26).

The approximate body weights are provided for all the species.

The data in table 1 do not show any clear correlation between size and hemoglobin-oxygen affinities. If, however, certain species are ignored (i.e., hamster, harvest mouse, and Norway rat), the remaining data suggest that such a relation may exist. This correlation is far from being exact and is presented here only to shed light on the hypothesis submitted by Schmidt-Nielsen and Gjönnes ('52). The author must point out, however, that this investigation employed dilute hemoglobin solutions, while Schmidt-Nielsen's hypothesis referred only to whole blood.

Figure 1 presents several selected dissociation curves. It includes the extremes and certain intermediates of the curves established during this investigation at pH 6.8 at a temperature of 37.0°C. Note that the hamster and human curves lie so close to each other that it was not practicable to draw them separately.

Figure 2 indicates a measurable difference between the dissociation curves of the white and Norway rats at pH 6.8. A standard deviation of regression was determined for these two curves, and they were found to be separated along the vertical axis by 2.5 standard deviations. In view of this fact and the additional fact that 4 separate curves were established for as many white rats and 4 curves for three Norway rats, the author believes this difference to be real even though it is near the resolving power for this technique. Anson et al. ('24) also reported subspecific differences in oxygen dissociation curves. It is interesting in this respect to note (fig. 2) that there was no measurable difference between the curves of the white mouse and house mouse.

## SUMMARY

Oxygen dissociation curves were determined for 15 species of mammals using dilute hemoglobin solutions at 37.0°C. and

pH 6.8. Of these species, 12 were true rodents; the remainder included the human and one species each of rabbits and shrews. Additional curves were established at pH 7.2 for 6 of these species in order to indicate the degree of the Bohr effect. The following conclusions were discussed.

1. There are considerable variations in hemoglobin-oxygen affinity even among species closely related phylogenetically and having similar habits and habitats.

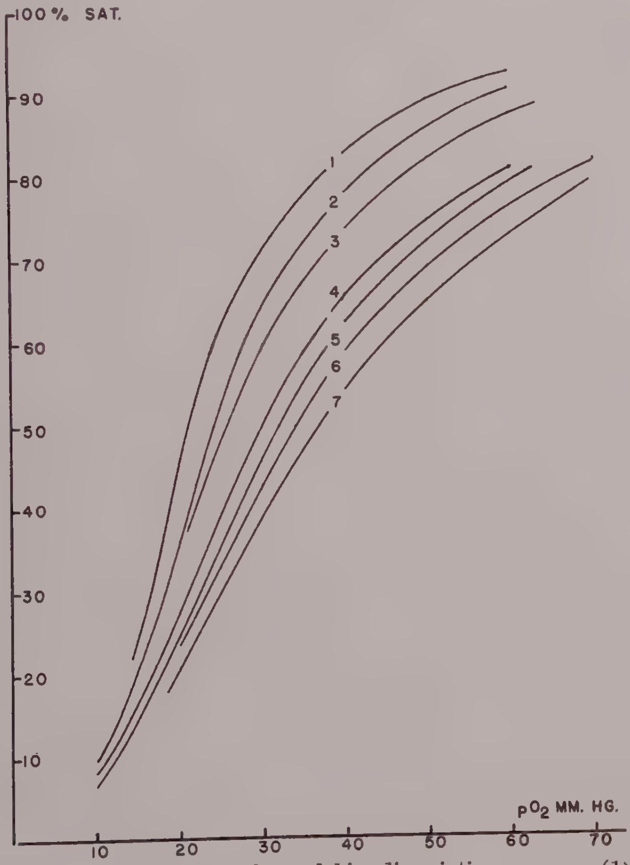

Fig. 1 Comparison of oxygen-hemoglobin dissociation curves. (1) Human.* (2) Rabbit. (3) Gray squirrel. (4) Flying squirrel. (5) Harvest mouse. (6) Pine mouse. (7) Golden mouse. Temperature 37.0°C. pH. 6.8.

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* The human and hamster curves are so close together that, for practical purposes, they can be considered identical.

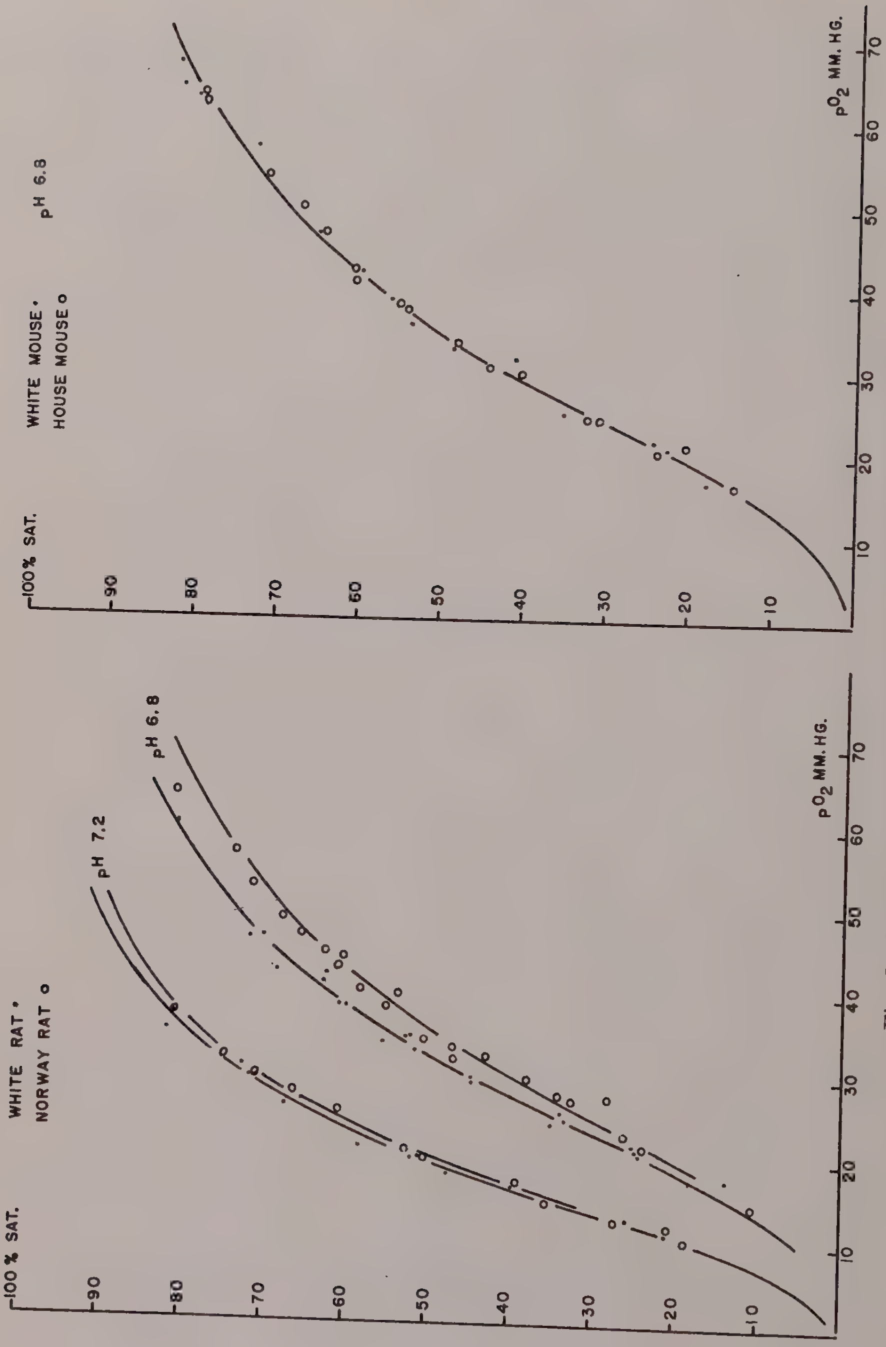

Fig. 2 Oxygen-hemoglobin dissociation curves. Temperature 37.0°C.

2. Rodents possessing hemoglobins with low oxygen affinity tend to show greater Bohr effects than those possessing hemoglobins with higher oxygen affinity.

3. Only a rough correlation between body weight and hemoglobin-oxygen affinity was found for the species of mammals studied in this investigation.

4. There may be small subspecies differences in hemoglobin-oxygen affinity since the dissociation curve of the white rat is slightly to the left of that of the Norway rat.

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# THE EFFECTS OF X RAYS ON DIVISION RATE AND SURVIVAL OF TILLINA MAGNA AND COLPODA SP. WITH AN ACCOUNT OF DELAYED DEATH[1]

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THREE FIGURES

A number of ciliates have been the objects of radiation study. The work to be described here was undertaken to extend the species range of these investigations. Both *Tillina* and *Colpoda* belong to the family Colpodidae. They differ from *Paramecium,* on which more work with radiation has been done, especially in that vegetative multiplication is by multiple division in cysts instead of by binary fission in the free-living condition. The investigation has revealed a form of delayed death which differs considerably from those previously described for x-irradiated cells.

## MATERIALS AND METHODS

*Tillina magna* and *Colpoda* sp. were excysted from soil collected in 1944 from a dried puddle in Sparrow's pasture near Chapel Hill, North Carolina. They were grown in lettuce solution inoculated with *Aerobacter aerogenes.* The strain of *Tillina* was amicronucleate whereas the strain of *Colpoda* was micronucleate. The *Colpoda* was a large form but specific identification has not yet been made. All the animals for a given experiment were derived from a single animal.

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[1] Work done under Contract no. W-7405-eng-26 for the Atomic Energy Commission.

[2] Work done while on leave of absence from Agnes Scott College, Decatur, Georgia.

The source of x rays was a G. E. Maxitron 250 operated at 250 kvp. and 30 ma. The tube had a thin beryllium window and no filtration was added. The intensity at 15.4 cm from the target, the position used, was about 16 kiloroentgens (kr) per minute. The output was measured before each exposure as described by Kimball, Geckler, and Gaither ('53).

Animals to be used were selected to be in nearly the same part of the division cycle and were washed through 6 changes of culture medium. They were then put in groups of 30 to 45 in a hanging drop on a thin sheet of mica inverted over the top of a plastic dish which contained a small amount of water to prevent drying. As soon as possible after exposure the drop was flooded with fresh culture fluid; and the animals were then transferred individually to fresh culture fluid to avoid, as far as possible, the effects of irradiated medium.

In the early phases of the work, the treated animals were observed frequently in the hours immediately following irradiation. In all work the descendants produced by each treated animal were counted the next day, and a single individual transferred to fresh medium to continue the line of descent. This procedure was repeated daily until the end of the experiment.

## EARLY VISIBLE EFFECTS

Tillinas exposed to 300–400 kr were dead by the end of the irradiation. When the mica cover slips on which they had been exposed were examined immediately after removal from the x-ray machine, the position of the body of many animals could be recognized by a little mass of material. The cell membrane, cilia, etc., appeared to be destroyed but parts of the cytoplasm seemed coagulated and did not flow into the culture fluid.

A dose of 200 kr killed most of the exposed animals immediately. In those few which survived, changes were apparent in both morphology and behavior. The size was sometimes diminished by an extrusion of cytoplasm. The dorsal knob was obliterated and its position marked by a large vacuole. The prominent semilunar curve of the cytopharynx and oral groove seen from the ventral surface either disappeared or became very indistinct. The animals lost their usual contours and took on a spherical shape. Cyclosis was very slow and the movement of gastric vacuoles seemed to be arrested. Contractile vacuoles sometimes continued to function slowly. The cilia remained intact and the animal swam slowly in small circles. This is the behavior pattern of animals about to encyst and does indeed result after a few hours in a kind of cyst. A few animals exposed to these doses have been observed to divide, often irregularly, but sooner or later they go into a kind of resting cyst and die. These cyst could not be excysted by the usual procedures and were visibly abnormal in that their membranes were thin and irregular and were encased in a thick scum of bacteria.

A dose of 100 kr was not immediately lethal. The animals showed the same general immediate responses as the survivors of higher doses, but the changes were less marked, and the animals returned to normal form and divided. Animals exposed to 50 kr appeared normal before the end of the first day, and those exposed to 25 kr showed no obvious changes.

## EFFECTS ON DIVISION RATE AND THE DIVISON PROCESS

Another measurable effect is a decrease in the rate of division. The number of animals was recorded each day, and from this record the number of divisions per day could be ascertained. However, a two-day average is a better measure because *Tillina's* usual rate is such that if one division event (encystment and division into two or more parts) occurs on one day, two are likely to occur the next, and vice versa. This measure for the first two days after irradiation is plotted against dose in figure 1. The points are based on averages of from 88 to 184 irradiated animals.

Usually the process of cyst formation immediately precedes two macronuclear and cytoplasmic divisions, giving rise to 4 daughter cells. However, even in the controls, irregular numbers of daughter cells were sometimes found. Thus it was practicable only to determine the number of daughter cells formed, not the number of encystments. ''Divisions'' as used in the rest of the paper will mean doublings in the number of animals while the term ''division event'' will be used for the process of division cyst formation and multiple division therein. In some instances, the smaller size of the individual used for transfer indicated that it had undergone one or two more divisions than some of its sisters. In this case, the estimated number of divisions undergone by the transferred animal was recorded and was used in calculations.

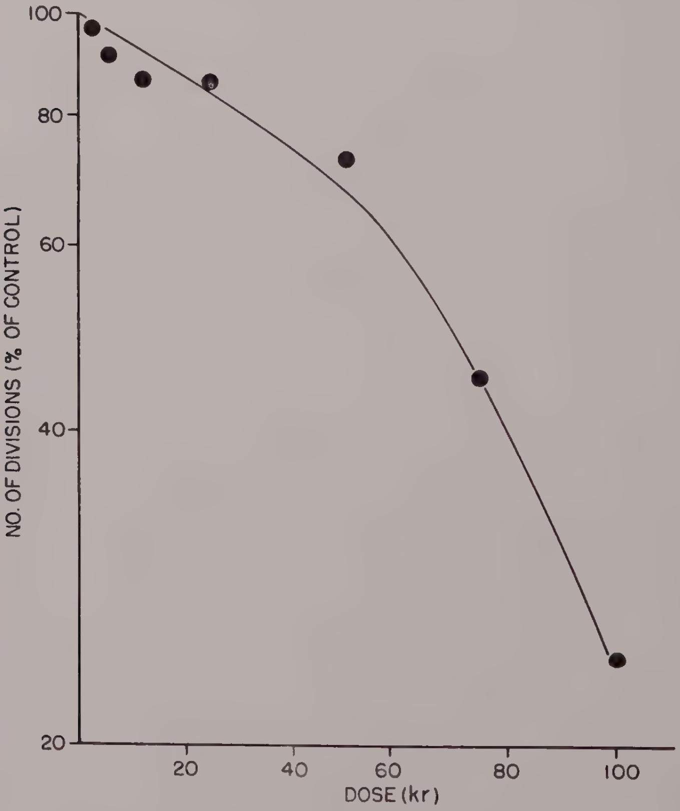

Fig. 1 Number of divisions in the first two days after irradiation expressed as a percentage of the control plotted against dose in kiloroentgens. Each point is the average of all experiments with *Tillina* at the dose in question.

These experiments indicate that radiation affects the mechanism of control of the number of divisions occurring during one division event. The usual division in *Tillina* results in 4 equal-sized daughters. In 67 of 88 cases following 75 kr and in 45 of 58 following 100 kr, the first division event resulted in two daughter cells whereas all the controls gave rise to the usual 4. Lower doses were often followed by the production of irregular numbers such as 3, 5 and 6.

Another effect was the production of animals of abnormal form. In lines which were later to die, such monsters were not uncommon.

### *Delayed death*

As the daily isolations of animals descended from irradiated animals continued beyond the first two-day period, many lines which at first appeared perfectly normal and had resumed a nearly normal division rate became abnormal, encysted and died. In this paper, death usually means the formation of an abnormal-appearing cyst from which excystment did not occur during a number of days. Some of these cysts were examined closely and found to contain the disintegrated remains of the animal. Occasionally no animal was found in a depression, presumably because it disintegrated without encysting or was not transferred. These cases were also counted as deaths but were rare.

The procedure in the daily isolations, when there was a choice, was to pick the most normal-appearing animal for transfer so that the effect of the radiation would be minimized rather than exaggerated. No adequate procedure for random selection was found. In spite of this choice, in many lines abnormal animals appeared and died. Animals were said to have died at a certain division if they completed that division but no further divisions before death. The rate of death, plotted in figure 2, was very high in the period from 4 to 14 divisions after irradiation and then leveled off to a more or less constant low rate. This rate was the same as or only slightly higher than the control rate. The reason for the death in the controls is not clear.

Four types of death can be distinguished provisionally: (1) death before the first division, (2) death between the 4th and 14th division, (3) death due to irradiation but occurring at a constant rate and continuing indefinitely, and (4) control death also occurring at a constant rate and continuing indefinitely.

Control death occurred in all experiments although the rate appeared to be somewhat different in different ones. The

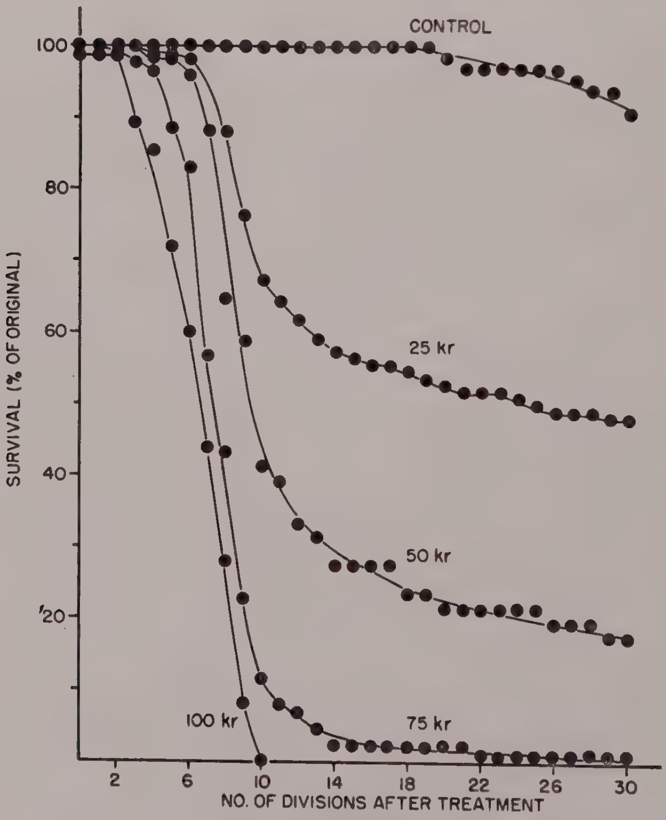

Fig. 2 Percentage survival plotted against the numbers of divisions after treatment for several different doses of x rays. All data are from a single experiment with *Tillina.*

period without death in the control in figure 2 is a fortuitous result of the small sample and was not found in some other experiments. The third type of death appears to occur in the 50-kr group shown in figure 2 and is also suggested by some other experiments. Inasmuch as low rates of death cannot be determined accurately when few surviving lines are available, this type of death cannot be considered to be established.

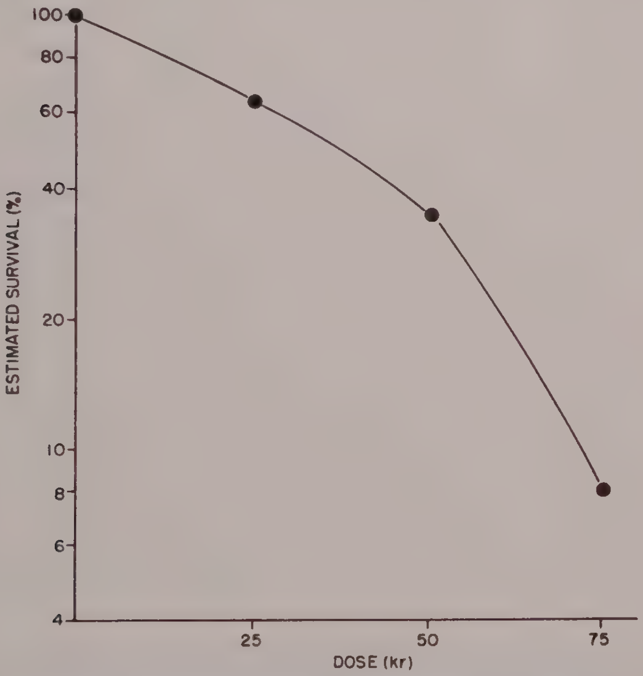

Fig. 3 Percentage survival, estimated by extrapolating the tails of the curves in figure 2 to 0 divisions, plotted against dose.

The dosage relations for the second type of death are set forth in figures 2 and 3. The average number of divisions to death decreases slightly with dose but the percentage of lines which die during the period increases rapidly with dose. This is shown in figure 3 in which the percentage survival is plotted on a logarithmic scale against dose. The percentage survivals were found by extrapolating the tails of the curves in figure 2 to 0 time to eliminate the constant rate death.

A similar experiment with *Colpoda* in which 198 isolation lines were exposed to doses ranging from none to 75 kr showed a different pattern of response. There were lines in which the division rate was unaffected, others in which it was markedly affected but in which recovery of normal rate was apparently in progress at the end of the experiment, and still others in which division rate was low and death finally occurred. In general, the lines which eventually died did not approach normal division rate even for one day, thus differing from *Tillina.* Furthermore, lines which divided slowly for some days but then appeared to recover were fairly numerous with *Colpoda* but not with *Tillina.*

Still further features of the delayed-death phenomenon are shown in tables 1 and 2. Table 1 shows the average daily divisions for the controls and 50-kr group for two sample experiments, one with *Tillina* and one with *Colpoda.* The irradiated lines are divided into categories according to their fate. In *Tillina,* it is clear that all lines suffer a reduction of division rate on the first day but only those which die show a reduction in the next several days. It is to be noted that only lines which divided on the day in question or divided on some later day are included in the averages. This was done because most animals died in cysts, and it is impossible to determine the day of death of a cyst with certainty. The lines which eventually died survived at least through the third day, and the average number of divisions on this day was only slightly less than the controls. Following the third day there was an irregular decline in rate terminating in death. The lines which survived 12 days nonetheless showed a terminal drop in average division rate, due almost entirely to a few lines with quite low rates. It is not clear whether these would eventually die or would survive with a permanent depression in rate.

Table 2 shows that it is possible to predict with rather good accuracy the fate of a line from its division rate in the

TABLE 1

*Sample experiments with Tillina and Colpoda*

| DOSE | KIND OF LINE | ITEM [1] | NUMBER OF DAYS AFTER IRRADIATION | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *kr* | | | | | | | | | | | | | | |
| | | | | | *Tillina* | | | | | | | | | |
| Control | All lines | D | 3.0 | 3.6 | 3.8 | 2.7 | 3.7 | 3.7 | 3.2 | 3.2 | 3.1 | 3.2 | 3.0 | 3.4 |
| | | N | 57 | 57 | 57 | 56 | 56 | 56 | 56 | 56 | 56 | 49 [5] | 47 | 45 |
| 50 | Died within 12 days [2] | D | 1.4 | 2.4 | 3.1 | 1.8 | 2.5 | 2.1 | 2.2 | 1.0 | 1.5 | 1.0 | .. | .. |
| | | N | 34 | 34 | 34 | 25 | 15 | 7 | 5 | 4 | 4 | 2 | 0 | 0 |
| | Survived 12 days | D | 2.0 | 3.8 | 3.2 | 2.7 | 3.5 | 3.2 | 3.1 | 2.4 | 2.7 | 2.8 | 2.4 | 2.2 |
| | | N | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 24 [5] |
| | | | | | *Colpoda* | | | | | | | | | |
| Control | All lines | D | 3.5 | 4.4 | 4.9 | 4.1 | 4.5 | 4.2 | 3.8 | 4.0 | | | | |
| | | N | 60 | 59 | 59 | 59 | 58 | 58 | 58 | 58 | | | | |
| 50 | Died within 8 days [2] | D | 2.6 | 2.4 | 2.8 | 2.0 | 3.0 | .. | | .. | | | | |
| | | N | 5 | 5 | 5 | 1 | 1 | 0 | 0 | 0 | | | | |
| | Low division [3] | D | 2.4 | 3.0 | 1.6 | 1.8 | 2.7 | 2.4 | 2.2 | 2.5 | | | | |
| | | N | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | | | |
| | High division [4] | D | 3.8 | 4.0 | 5.3 | 3.9 | 4.5 | 4.3 | 3.9 | 4.1 | | | | |
| | | N | 37 | 37 | 37 | 37 | 37 | 37 | 36 [5] | 35 [5] | | | | |

[1] D = Average divisions in lines which divided on day in question or divided on some later day before death.
N = Number of lines on which the average was based.

[2] Five colpodas but no tillinas died without dividing and are not included.

[3] Less than 30 divisions in 8 days.

[4] Thirty or more divisions in 8 days.

[5] Accidental losses.

first two days; i.e., before it reaches its highest division rate if it finally dies. Thus the change which eventually leads to death usually expresses itself immediately after irradiation. Death is delayed, but not the changes leading to it.

Table 1 shows the very considerable differences between *Colpoda* and *Tillina*. Colpodas which died also had a low division rate from the start but showed little if any sign of a recovery of normal rate before death. A considerable number of lines did not die during the 8 days of the experiment although they did show a persistently low division rate. Some of these lines gave evidence of recovery before the end of the experiment, but others may have been destined to die. The remainder of the lines appeared to have been totally unaffected even in the first day's division. Finally, a number of lines died before the first division at 50 kr, a rare event for *Tillina*.

TABLE 2

*Numbers of divisions in the first two days after irradiation in lines which died and lines which survived, Tillina, 50 kr*

| NUMBER OF DIVISIONS | NUMBER OF LINES HAVING THIS NUMBER OF DIVISIONS | |
|---|---|---|
| | Died within 12 days | Survived 12 days |
| 0 | 2 | 0 |
| 1 | 0 | 0 |
| 2 | 1 | 0 |
| 3 | 10 | 0 |
| 4 | 12 | 3 |
| 5 | 0 | 0 |
| 6 | 7 | 21 |
| 7 | 0 | 1 |

## DISCUSSION

The effects of x rays on division rate and survival for the first day after irradiation are not unlike those reported for other ciliates, e.g., Kimball, Geckler, and Gaither, '53 for *Paramecium*. However, somewhat lower doses seem to be required. Interference with the division process as shown by the low and irregular number of divisions in the cysts and by monster formation appears to be fairly common. Monster formation has seldom been found after x irradiation of *Paramecium* in this laboratory although it has been reported by others (for review see Wichterman, '53; and Kimball, '54). The irregularities in the division processes within the cysts do not, of course, have parallels in ciliates which divide by binary fission. It is tempting to speculate that the relatively high x-ray sensitivity of *Colpoda* and *Tillina* is connected in some manner with their special division mechanism.

Delayed death in *Tillina* has some similarities to delayed death after ultraviolet irradiation of *Paramecium aurelia* (Kimball, Geckler, and Gaither, '53), but the differences are considerable. Delayed death has not been found after x irradiation of *Paramecium aurelia*; but Kimball, Geckler and Gaither ('53) give reasons for thinking that it would be found if sufficient paramecia survived the first division at high doses and that it would be similar to that found with ultraviolet. If this is so, the doses required for *Paramecium* must be greater than 300 kr, whereas appreciable delayed death occurs in *Tillina* given only 25 kr.

In addition to the dose required, delayed death in *Tillina* differs from that in *Paramecium* in several other important respects. Almost all tillinas which eventually die suffer a large depression in division rate in the first few days whereas those which survive show much less depression. In *Paramecium,* no such correlation exists. The surviving tillinas show very little effect beyond the first two days whereas surviving paramecia undergo a marked depression in division rate coincident with the period in which others die. In fact, the impression is gained that only chance determines whether or not a particular animal will survive this period of depression. The death is a more or less accidental by-product of the depression which affects all lines. In *Tillina,* the changes which lead to death affect only a part of the lines at doses below about 75 kr, and the changes almost invariably lead to death. Finally, the average number of divisions to death is not the same in the two species. In *Paramecium* the marked depression with which death is associated usually comes after the third or 4th division, and recovery of the survivors is essentially complete by the 6th division. In *Tillina,* the death is spread over the period between the 4th and 14th divisions. Thus, though the changes which lead to death have already made themselves apparent in the first few divisions, the animals often survive for many divisions longer and may even recover the normal division rate in the interim.

The apparent dichotomy between the lines which show a large delay in division and die and those which show only a small delay and survive may be due to the existence of two groups of animals of different sensitivity. Although an effort was made to avoid this by selecting animals somewhat alike in size and by using a single clone, such procedures do not ensure homogeneity. However, the smooth curve in figure 3 makes this interpretation unlikely. If there were really two groups of distinctly different sensitivity then this should be reflected by a sharp break in the dose curve. The hypothesis that irradiated animals consisted of individuals with a series of different sensitivities would account for a more or less smooth dose curve but would still require that the response of any one animal be almost an all-or-none matter since so few cases which could properly be called intermediate, i.e., low early division rate and survival or high early division rate and death, were found.

The best known mechanism for producing death in some cells and not in others given the same dose is the induction of lethal mutations. In the amicronucleate *Tillina* used in this study, this would mean either macronuclear mutations or mutations in some self-reproducing cytoplasmic entity. The latter, if conceived as mutations among numerous randomly distributed self-reproducing particles, would have the same properties, for present purposes, as macronuclear mutations and so need not be considered further. The macronucleus of *Paramecium* has been shown to contain many sets of chromosomes either held together in sets (subunit hypothesis) or scattered as individual chromosomes (polyploid hypothesis). The macronucleus of *Tillina* is presumably similar. Sonneborn ('49) and Kimball ('53) may be consulted for discussion. In any case, it seems probable that the subunits or chromosomes are distributed at random at division since there is no recognized mechanism for precise distribution. Mutations affecting one or a few subunits or chromosomes might then segregate out in succeeding divisions and finally reach sufficiently large proportions to kill the cell. Kimball and Householder ('54) have shown by theoretical calculations that certain simple forms of this hypothesis do not fit the present data. However, the simple theory could probably be modified to fit the data if it were not for the more serious objection of the close connection between the division rate of the first two days and eventual death. It is difficult or impossible to see how a mutation in only one or a few of many homologous chromosomes could have an immediate effect on division rate from which partial recovery occurred to be followed almost inevitably by death. Such a rapid effect on the division rate is most improbable from present understanding of gene action; and random segregation of mutations combined with the choice of the best animal each day should have ensured the elimination of some mutations which originally affected the division rate.

Thus the two hypotheses which might have accounted for a kind of all-or-none phenomenon are unlikely. This leaves delayed death in *Tillina* in the unanalyzed residue of radiation-induced changes which are not attributable to known genetic effects. However, we know of nothing in the cell which could furnish a material basis for an all-or-none phenomenon in such cases. We can only point out that our data strongly suggest that such a phenomenon can occur and emphasize that considerable periods of time and numbers of divisions may intervene between the original damage and the final death of the cell.

## SUMMARY

1. An amicronucleate strain of *Tillina magna* and a micronucleate strain of a large species of *Colpoda* were exposed to doses of x rays ranging from 25 to 400 kr.

2. Doses of 200–400 kr were without exception lethal to *Tillina,* and a description is given of some of the visible changes in structure associated with rapid death.

3. At lower doses, the division rate in the first two days was decreased and abnormalities in the process of multiple division in cysts occurred.

4. In *Tillina,* a part of the animals recovered nearly the normal division rate but eventually died after 4–14 divisions following irradiation. The percentage of lines showing this delayed death is a function of dose, but the number of divisions to death decreases only slightly as dose increases.

5. The lines which eventually died differed from those which survived in having, for the most part, a lower division rate during the first day after irradiation despite the fact that most of them subsequently recovered nearly the normal rate before going into the final decline leading to death.

6. This is interpreted to mean that the delayed death could not be due to segregation out of lethal mutations in the macronucleus as a result of random segregation at division. The possibility that the irradiated tillinas consisted of two classes of different sensitivity also seems unlikely. Thus this case gives evidence for a kind of all-or-none effect on the cell which is not mutation as ordinarily understood.

7. *Colpoda* responds in some ways like *Tillina* but differs in several important respects.

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# INTERNAL HYDROSTATIC PRESSURE OF THE FUNDULUS EGG

## II. PERMEABILITY OF THE CHORION[1]

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TWO FIGURES

### INTRODUCTION

By means of a micropipette connected to a specially built mercury manometer, the development of an internal hydrostatic pressure within the fundulus egg when activated either by micropuncture or by successful insemination was recorded, and the results described in a previous paper (Kao and Chambers, '54). Evidence was there presented that the pressure developed concommitantly with the disappearance of small vacuoles, the so-called platelets, in the cortex of the egg proper and with the formation of a perivitelline space which separated the egg proper from the surrounding chorion. Both the perivitelline space and the pressure increased gradually during the first hour and then stabilized at a steady level thereafter for long periods of time. Simultaneous with the enlargement of the perivitelline space, a clear jelly-like colloid accumulated therein, which under normal conditions did not pass through the chorion. A close relation was found between the size of the perivitelline space and the magnitude of the pressure. The latter when once stabilized could be eliminated either by tearing a hole in the chorion whereupon the colloid in the perivitelline space escaped into the surrounding medium; or by immersing the egg in a hypertonic sucrose solution which induced a simultaneous reduction in the size of the perivitelline space. On the basis of these results, together with the sequence of events accompanying the development of the perivitelline space and internal pressure, it was concluded that, upon the disintegration of the platelets, a colloidal material was released just exterior to the egg proper. There, retained by the external chorion, it slowly imbibed water, causing an enlargement of the perivitelline space, and a compression on the egg proper with a resultant increasing pressure. The mechanism of the formation of the perivitelline space is similar to that proposed by Boguchi for the trout egg ('30).

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To support this view, it is necessary to show that other substances normally occurring in sea water, especially the osmotically more active ions, contribute little towards the formation and maintenance of the internal pressure. Using the technique of pressure determination, the latter has been ascertained by establishing the absence of an osmotic pressure induced by changing the ionic concentration of the external environment. This not only shows the insignificant role of the ions in maintaining the internal pressure, but also shows a more important point, namely, that water and ions can traverse the chorion of the fundulus egg, thus negating existing statements that the chorion of the fundulus egg is impermeable to water or ions (cf. Kao and Chambers, '54). In this paper results are presented to show the effect on the internal hydrostatic pressure of increasing or decreasing the ionic concentration of the external environment, and also the effect of some specific ions on the development and maintenance of the pressure. Preliminary reports have been published by Buno and Chambers ('47) and by Chambers, Chambers and Kao ('51).

The effect of media of different ionic concentrations on the freezing point depression of the whole fundulus egg has been studied by Loeb and Wasteneys ('15). They concluded that the chorion was impermeable to water or salts, and attributed the change of the Δfp they found to be due to salts trapped within the meshes of the chorion. In view of our recent evidence that the internal hydrostatic pressure is present only when there is a distinct perivitelline space which is apparently formed with some movement of water, the data of these earlier workers are reanalyzed, and a conclusion directly contradictory is reached. The two conclusions and the basis of disagreement are discussed.

## METHOD

Pressure was recorded in the same manner as described in our previous paper, by inserting a glass micropipette connected to a manometer into an unfertilized fundulus egg. As indicated previously, the puncture itself served as an effective means of activation, and initiated the formation of an internal pressure, generally separable into an early rising stage and a subsequent plateau stage. The rising stage lasted from the time of puncture to about the end of the first hour during which the internal hydrostatic pressure increased along a sigmoidal curve from an initial negative value. The second or plateau stage directly followed the rising stage and showed a stabilized pressure of about 150 mm Hg. for a protracted period. In the experiments reported here, the eggs were exposed during both stages of hypo- or hypertonic environments of various concentrations. The changes in tonicity of the external medium were affected by replacing an appropriate amount of sea water from the dish containing the egg with distilled water or with specific concentrations of salt solutions. During the rising stage of the pressure, the changes of the surrounding solutions were made not earlier than 15 minutes after puncture, because changes made before this period tended to disrupt the plug which was formed at the site where the shaft of the micropipette penetrated the egg. Any disruption of this plug was liable to induce leakage, thus vitiating the experiment. For this reason also, all changes of the medium were performed in such a manner as to minimize any convection current in the surrounding solution.

## RESULTS

### *Effect of electrolytes during the rising stage of the pressure*

The internal pressure of the unfertilized egg activated by being punctured in sea water increased progressively as described in our previous paper. Figure 1 presents the time course of the internal pressure in 4 experiments in each of which the medium was changed 15 minutes after the puncture was performed. Curves, A, B and C represent the effect of hypotonic media of 10, 25 and 50% sea water respectively. Regardless of the dilution, the change from 100% sea water to a more dilute environment occasioned an immediate rise of the pressure. This is clearly manifested by a hump arising from a smooth curve which averaged about 90 mm Hg. in height just before the change was made. The sudden rise caused by the hypotonic media reached a peak within two–three minutes, attaining an additional 20–30 mm Hg. It was then followed by a more gradual fall spreading over 5–6 minutes when the pressure returned to a level that would have been expected if no intervening dilution had been made. From then on the pressure continued to increase just as it does in sea water, reaching an average of 150 mm Hg. at about 50 minutes.

Curve D is of an experiment in which an egg was stripped from the fish directly into 50% sea water, followed immediately by puncture. The process of activation and the recorded pressure were similar to those in sea water. After immersion for 15 minutes in 50% sea water the external medium was changed to 100% sea water. This caused a sharp fall of pressure of about 20 mm Hg. which persisted for 5 minutes as indicated by the dip in the curve. The fall, like the rise of pressure in the preceding curves, reached its lowest value 82 mm Hg. within two–three minutes. It was then followed by a more gradual increase reaching the expected level in a longer time. At 50 minutes the pressure stabilized at 148 mm Hg.

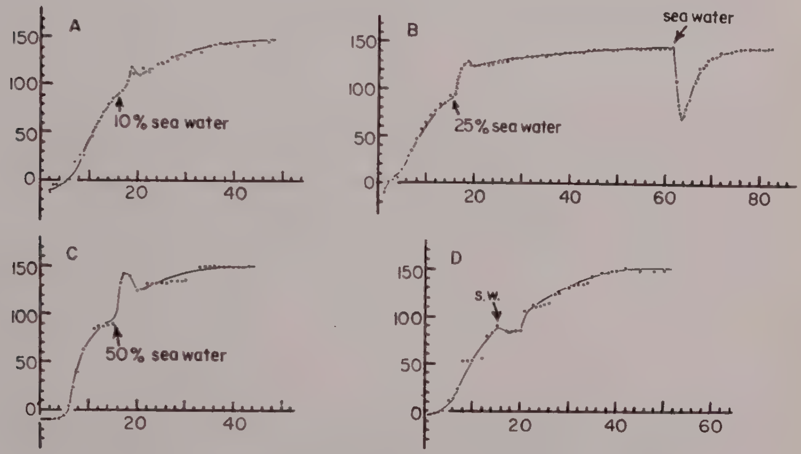

Fig. 1 Effect of dilutions of sea water on the internal pressure in the rising stage. Ordinate: pressure in mm Hg.; abscissa: time in minutes. A, B, C, eggs stripped into sea water and at time indicated the dilution of the environment was accomplished. The eggs remained in the hypotonic environment through the rest of the experiment except in B where it was immersed again in sea water at 61 minutes. D, egg stripped into 50% sea water which was replaced at time indicated by 100% sea water. Note the respective transient rise and fall of pressures.

### *Effect of electrolytes during the plateau stage*

Curve B (fig. 1) presents an experiment in which at 15 minutes after puncture the medium was changed from 100% to 25% sea water. A transient rise in pressure caused by the dilution occurred, and at 50 minutes the pressure stabilized at 145 mm Hg. with the egg remaining in 25% sea water. During the plateau stage, at 61 minutes after puncture, the medium was changed from 25% back to 100% sea water, which was at this time hypertonic to the whole egg. As in curve D described above, the pressure fell rapidly and reached its lowest reading of 70 mm Hg. within 2½ minutes; and then rose again to the usual level of 150 mm Hg. within 14 minutes.

Similar results were obtained with calcium chloride, sodium chloride, and with sea water, indicating that the effects noted were those of hypo- and hypertonicity of the media, and not of any specific salt used. The experiment with $CaCl_2$ is as follows: an egg was treated as usual in sea water, and exhibited the usual pressure. The part of the curve shown began at 60 minutes. At 70 minutes (fig. 2) the sea water medium was changed to 0.17 M $CaCl_2$ which is half isotonic to Woods Hole sea water. This was followed by an immediate rise in pressure and a slower return to the original level. After the pressure has stabilized for 10 minutes, the hypotonic $CaCl_2$ solution was replaced by sea water. This, being hypertonic, induced an immediate fall of 45 mm Hg., followed by the usual more gradual return to the original plateau level. The procedure was repeated on the same egg after 12 minutes during which the plateau pressure had been maintained. As

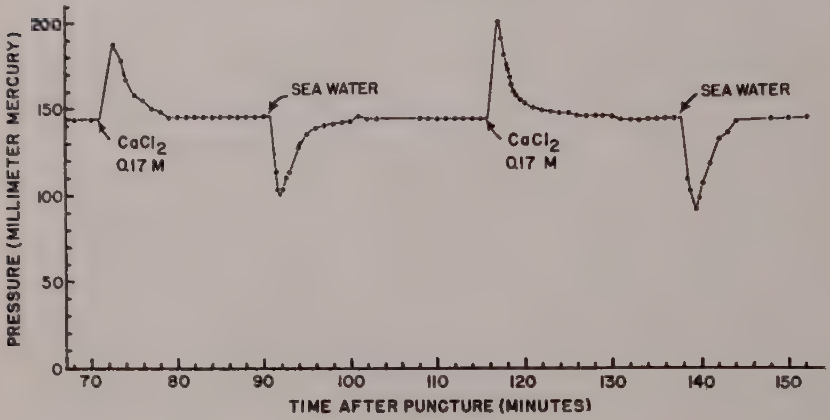

Fig. 2 Effect of electrolytes on the internal pressure during the plateau stage. The egg was punctured in sea water and exhibited the usual development of pressure. $CaCl_2$ 0.17 M is half isotonic to Woods Hole sea water. Note the transient changes of pressure and return to the original level.

shown in figure 2 the same rise and fall with subsequent return to the original level was observed.

### *Effect of NaCl and $CaCl_2$*

While the effects of these two salt solutions on a stabilized pressure showed little specificity of action, a marked difference of action was observed on the initial development of the internal pressure. For this purpose, eggs were stripped from female fish directly into isotonic NaCl or into $CaCl_2$ solutions separately and were punctured in the usual manner. In NaCl, the platelets disappeared tardily and incompletely. The internal hydrostatic pressure under these conditions had a very slow rising phase, and generally reached a lower level than the 150 mm Hg. observed in sea water. In $CaCl_2$ the converse was true. The platelets disappeared so rapidly that the eggs were already activated within the two to three minutes' time generally required for manipulations before a puncture could be performed. A significant pressure was often in existence at the time of puncture, and was artificially increased by the exertion of mechanically applied pressure by the micropipette on the tough chorion. In all the eggs which remained in isotonic $CaCl_2$ for some time, the surface of the egg proper became slightly granular and very brittle. Any minute movement of the pipette would cause extensive rupture of the egg proper. This hardening action of $Ca^{++}$ on the external material of the egg proper is in agreement with Chambers' findings on other types of cells and on the intercellular cement of the capillary bed (Chambers, '49). A similar effect of $Ca^{++}$ on the activation process was described recently by Yamamoto ('54) for the egg of *Oryzias latipes* which did not undergo spontaneous activation in Ca-free medium.

### *Phenol red, HCl, and NaOH*

Eggs used in these experiments were stripped into sea water containing 0.2% phenol red and spontaneous activation was allowed to proceed. The medium was colored light pink because of the alkaline pH of 8.2 of the sea water. When the perivitelline space was fully developed, eggs were transferred to a dish of fresh sea water, and excess color washed off. When examined against a colorless background, it could easily be determined that the perivitelline space was colored light pink, the egg proper showing no color except for the natural cream color of the yolk. A few drops of HCl were then placed in the vicinity of the eggs. Immediately the color of the perivitelline content changed from pink to bright yellow. When NaOH was then added to the sea water, the yellow perivitelline space returned to the pink alkaline color. These color changes of the perivitelline contents clearly indicate free passage of hydrogen and hydroxyl ions through the chorion.

## DISCUSSION

As shown in a previous paper (Kao and Chambers, '54), the source of the internal hydrostatic pressure resides almost entirely in the perivitelline contents. The transient changes in pressure induced by hypo- and hypertonic solutions are undoubtedly also related to the perivitelline contents because of the rapidity of the events. They are to be interpreted as indications of passage of both water and salts rapidly through the chorion, much in the same manner as the sequence of volume changes occurring in a cell in a hypertonic solution of a penetrating substance. In hypotonic solution, the initial rise in pressure can be explained as caused by water entering the perivitelline space where some salts from a previous environment still remain. As water enters, no measurable volume change occurs with regard to the whole egg since this is enclosed by an inelastic chorion but an increased pressure develops within. The subsequent return of the pressure to the original level is to be looked upon as a manifestation of the exit of salts from the perivitelline space, the more gradual slope indicating a slower mobility through the chorion of salts than water. The converse occurs in hypertonic environments. There is first a fall and then a return of pressure to the original, signifying a rapid exit of water and a slower entrance of salts into the perivitelline space. In both cases a pressure stabilized at the original level following the transient changes must indicate that the ionic concentrations of the external environment and the perivitelline fluid have reached a new equilibrium. In other words, save for the colloidal material, the perivitelline content is the same as the external environment in its ionic composition. The constancy of the internal pressure in environments of widely different ionic concentration shows that ions are not important in maintaining the internal hydrostatic pressure which is to be ascribed only to the perivitelline colloid.

Thus, the fundulus egg resembles the trout egg in having a porous chorion and a perivitelline fluid similar to the fluid of the external environment. In the trout egg this was established by Svetlov ('29) who found that changes of the external environment altered the freezing-point depression of the whole egg by an amount attributable only to replacement of the perivitelline content by the external fluid. Earlier work of Loeb and Wasteneys on the fundulus egg ('15), using the same technique, however, led these workers to a conclusion that the chorion of this egg was impermeable to either water or salts. This conclusion relied heavily on Loeb's observation ('12) that fundulus eggs remained floating in hypertonic salt solution for days. To Loeb this meant that there was no alteration of the density of the egg and hence no exit of water, the barrier being attributed to the chorion. Data presented in this paper of rapid and transient pressure changes indicate that although there is no net exchange of water as shown by a return to the original pressure, movements of water and salts through the chorion actually do occur in both directions with rapidity. The sustained floating of fundulus eggs, therefore, is not a sign that the chorion is impermeable to water.

Loeb and Wasteneys ('15) found some changes in the freezing point of the whole egg among the lots in solutions of different ionic concentrations. On the basis of the floatation observation these changes were interpreted as caused by salts trapped within the meshes of the chorion. Their data are presented and analyzed in table 1 in the light of our recent findings. It has been shown previously (Kao and Chambers, '54) that the perivitelline space when fully formed occupies about 40% of the total volume of the whole egg. By assuming that only 40% of the total constituents are free to exchange with the environment, values of freezing-point depression are calculated. As shown in table 1, these values are almost identical with those observed by Loeb and Wasteneys. Such remarkable agreement is not merely fortuitous when one considers the fact that the chorion is quite porous as indicated by the rapid pressure changes. Therefore, Loeb and Wasteneys' data actually indicate free passage of water and salts through the chorion.

TABLE 1

*Changes in freezing-point depression found by Loeb and Wasteneys compared with calculated values based on the thesis that the chorion is freely permeable to water and to electrolytes*

| A | B | C | D |
|---|---|---|---|
| ΔFP OF SEA WATER USED BY LOEB AND WASTENEYS TO WASH EGGS | 40% OF VALUE IN COLUMN A | EXPECTED ΔFP OF WHOLE EGGS (0.456 + COL. B) | ΔFP OF WHOLE EGG FOUND BY LOEB AND WASTENEYS |
| 0.52 | 0.208 | 0.664 | 0.66 |
| 0.55 | 0.220 | 0.676 | 0.68 |
| 0.67 | 0.268 | 0.724 | 0.72 |
| 0.68 | 0.272 | 0.728 | 0.75 |
| 0.80 | 0.320 | 0.776 | 0.77 |
| 1.04 | 0.416 | 0.872 | 0.88 |
| 1.93 | 0.772 | 1.228 | 1.27 |

A solution with Δfp of 0.76 was found by Loeb and Wasteneys to be isotonic with the whole fundulus egg. The expected values of Δfp are calculated by assuming that 60% of the total constituents do not exchange, and have a fixed Δfp. The other 40%, corresponding to the perivitelline contents, exchange freely, and have a Δfp of the environment. For each test condition, the proportion contributed by the non-exchangeable portion is fixed and equals 0.456. The contribution from the perivitelline content is shown in column B. The slight variation in two values (4th and last) may be due to the presence of a perivitelline space greater than 40%.

The assumption that only the perivitelline fluid is free to exchange with the environment is justified by the fact that the pressure always returned to the original level. It does not necessarily imply that the surface of the egg proper is invariably impermeable to water and salts. In a previous paper evidences were presented to indicate that the surface of egg proper is probably permeable to water under some conditions. Briefly stated these were: changes in the volume of the perivitelline space, either during the process of activation or as a result of immersion in a hypertonic sucrose solution, corresponded closely to a reciprocal change in the volume of the egg proper. It was concluded that under these conditions water exchange must occur between the perivitelline space and the egg proper. The return of pressure to the original level in salt solutions only indicates the porous nature of the chorion. The duration of the experiment herein reported, however, has never been long enough to permit any significant changes involving the egg proper to take place. An implication that the egg proper is impermeable to water and salt from the present data is therefore unwarranted. Nevertheless, the data do show that the chorion is much more permeable than the plasma membrane, a fact well within expectation.

The sustained floating of the fundulus egg in hypertonic solution of balanced salt solutions as observed by Loeb ('12) can only indicate a permeability barrier at the surface of the egg proper, and not at the porous chorion. This, however, must be verified by more direct evidence, such as isotope studies, before a conclusion is drawn that the plasma membrane is completely impermeable to water and salts. With trout eggs some evidence is available that diffusion of $D_2O$ through the plasma membrane is negligible (Krogh and Ussing, '37; Prescott and Zeuthen, '53). The implication, as has already been suggested by Ussing ('52) and by Prescott and Zeuthen ('53) is that the surface of egg proper of the trout egg is composed of a continuous lipoid phase in which water is only sparingly soluble, thereby supporting Gray's view ('32) about some static properties of the vitelline membrane [for difference of terminology cf. Gray ('32, p. 279) and Kao and Chambers ('54)]. This may also be the case with the fundulus egg, or other teleost eggs. The evidence, however, is not necessarily in conflict with our findings and conclusions. The determining factors are time and the activation (or water-hardening) process. Our work has been consistently performed on eggs freshly removed from female fish before any visible evidence of activation is present. At this early stage, there is always a negative pressure in the eggs for the first few minutes immediately before and during the beginning of the activation process (Kao and Chambers, '54). This negative pressure is to be explained as being due to a slight hypertonicity of the external sea water with respect to the egg interior. It indicates that water is readily lost to the environment in those few minutes. Upon activation the permeability of the surface of the egg proper rapidly decreases, a fact already indicated at 15 minutes after puncture when pressure returns to a predictable value after transient changes. Other workers have dealt with a later stage when activation is fully accomplished in both the fundulus and trout eggs. Their claims of impermeability to water in both cases are complimented by our finding that the permeability of the plasma membrane of the fundulus egg decreased rapidly within the first hour. This is supported by direct evidence with tracer studies in the case of the salmon egg which becomes impermeable upon activation (Prescott, personal communication). But as stated previously, our data do not permit the conclusion that the fundulus egg proper is completely impermeable to water.

An important and interesting question is raised by our observations, viz., the molecular reorganization of the plasma membrane during activation which changes the structure from one through which water passes freely to one through which water passes with difficulty. Perhaps the disintegration of the platelets releases some substance which either covers, so to speak, the external surface of the plasma membrane, or which initiates a sequence of reorganization therein. As a result of either, the plasma membrane would become resistant to through-passage of water. On the other hand, the sparing permeability may be a simpler process not necessitating any extensive reorganization of the plasma membrane, but may be one which is closely related to the volume of the perivitelline colloid. Recent evidences (cf. discussions in Jacobs, '52; Ussing, '52) indicate that water movement across the plasma membrane occurs as both a diffusion and a filtration process. The former entails solution of water molecules in the plasma membrane, whereas the latter is accomplished in bulk through pores therein. Water movement by filtration is generally established by osmotic gradients and is markedly affected by the friction offered by the pores in the plasma membrane. Reducing the pore size would increase the friction by a 4th power factor (Ussing, '52; Prescott and Zeuthen, '53); increasing the pore size would have the reverse effect. Our data indicate that when the volume of the perivitelline colloid does not change, as in cases when equilibrium can readily be established on the two sides of the chorion, the plasma membrane remains sparingly permeable. When the volume of the perivitelline colloid is decreased by hypertonic solutions of substances, such as sucrose, which crosses the chorion slowly, water movement through the plasma membrane by osmotic gradient is fairly rapid. An illustration of the latter is shown in preliminary experiments that in hypertonic sucrose solution, the internal hydrostatic pressure fell to zero or to a slightly negative value, the fall being accompanied by a sudden swelling of the egg proper which took up most of the perivitelline space. The increase in the volume of the egg proper tends to indicate that the perivitelline colloid maintains the sparing permeability of the plasma membrane under conditions in which its own volume is not altered. It may act simply by blocking the pores in the plasma membrane thereby reducing the effective pore size and increasing the resistance to water filtration. Or it may act by virtue of the compression it exerts on the egg proper. As indicated previously, in the presence of a perivitelline compression the egg proper decreases in volume as well as surface. The decrease of surface may occasion a closer packing of neighboring units of the plasma membrane thus decreasing the effective pore size, through which water filters. When perivitelline pressure is released, as in hypertonic sucrose solution, the egg proper swells initially. Only a slight swelling should be necessary to increase the pore size enough to allow entrance of water which will cause further swelling. Whatever the mechanism may be, the perivitelline colloid most probably plays a prominent role in maintaining the nature of the plasma membrane, and hence indirectly serves as an osmotic regulatory mechanism. Supporting evidence to this effect has been obtained by Yagle ('30) whose seemingly contradictory observation that dechorionated fundulus eggs were more permeable than whole eggs becomes plausible in the light of these suggestions.

## SUMMARY

The internal hydrostatic pressure of the unfertilized fundulus egg undergoes transient changes in environments of hypo- and hypertonic salt solutions. In hypotonic solutions the pressure rises rapidly and then returns to the original more gradually. In hypertonic solutions it first falls and then returns. These changes are interpreted as indications of passage of water and salts through the chorion. In hypertonic sucrose solution the pressure drops and remains at a low level for some time, while the egg swells initially but gradually shrinks again. A postulate is advanced that the perivitelline colloid plays a prominent role in maintaining the nature of the plasma membrane. The latter becomes sparingly permeable to water when the perivitelline colloid is in the fully imbibed state which exists when the activation process is completed.

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# HERITABLE NON-GENIC CHANGES INDUCED IN YEAST BY ULTRAVIOLET LIGHT[1]

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TWO FIGURES

A gene mutation involving a deficiency in the cytochrome system of yeast was produced by ultraviolet irradiation, (Raut, '53). This deficiency resulted in a complete, or almost complete, disappearance of all the cytochrome bands and a consequent inability of the cells to consume oxygen. In view of the reports of the effect of oxygen on the action of ultraviolet light, (e.g. Meyer and Miller, '52) the rate of kill of normal and mutant cytochrome-deficient strains with ultraviolet light was tested. No difference in the rate of kill of the two kinds of yeast was demonstrated, but as many as 50% of the surviving cells gave rise to cytochrome-deficient colonies when plated out on nutrient agar plates.

Further study of these cytochrome-deficient survivors showed that, although some were mutants, the bulk were "petites," which have been shown, (Ephrussi, et al., '49) to occur when self-reproducing *non-genic* elements are lost from normal cells.

## MATERIAL AND METHODS

The haploid strains irradiated were cytochrome-deficient mutant 103.1, normal 108.3, and a petite variant 108.3–P, isolated from a petite colony which appeared spontaneously

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on a plate of normal 108.3. Culture 108.3 was selected for irradiation because in this strain the buds are soon detached from the mother cell and form suspensions containing chiefly single cells. The cytochrome-deficient strains generally do not form clumps of cells.

As further insurance against clumping of cells, the suspension was prepared for irradiation by scraping the yeast off a two-day-old slant of nutrient medium,[2] grinding it against the dry wall of a sterile water blank with a sterile pipette and then suspending the cells in the water. After allowing the cells to settle in the tube for about 15 minutes, the upper portion of the cell suspension was pipetted into a small flask of sterile water. The cells in the suspension were counted in a haemocytometer and observed for clumps. Only 5% to 7% of the cells were in groups of two or three. No large clumps of cells were observed. A suitable dilution of this suspension was plated in triplicate to determine the viable count. The cell suspensions to be irradiated contained 1 to $2 \times 10^7$ cells per milliliter.

Five milliliters of the cell suspension were placed in each of a series of Petri dishes to be irradiated. Individual aliquots were irradiated separately for from 5 seconds to 90 seconds. During irradiation the plates were shaken very slightly by hand on the surface of the table to maintain an even distribution of the cells and to insure uniform exposure of all the cells.

The ultraviolet source was two 30-watt G. E. sterilamps mounted in parallel parabolic fixtures 66 cm from the irradiated surface. This yielded 14,000 ergs/cm$^2$/sec., with 95% of the irradiation at 2537 Å. A variable transformer on the line to the lamps was adjusted to maintain a constant reading on an ultraviolet photometer at the level of the irradiated suspensions. Following irradiation, the suspensions were suitably diluted and plated in triplicate on agar plates of nutrient medium. All operations after irradiation were carried out under a dim yellow light, and the suspensions were kept dark except during handling. The plates were incubated at 30°C., and colonies were counted after two days and again after a week. Normal and cytochrome-deficient colonies were classified by their appearance after a week's incubation. Normal colonies are rough, cream colored, and large; and the cytochrome-deficient colonies are smooth, white and smaller than the normals. In the first experiments, all the colonies classified as cytochrome-deficient were verified by streaking on no-sugar plates; in later experiments, only crowded colonies or slow growing colonies that were difficult to classify were verified by streaking on no-sugar plates. In preliminary experiments it was shown that although both normal and cytochrome-deficient cells produce colonies on nutrient medium plates, only normal cells produce colonies on plates of nutrient medium if the sugar is omitted. The normal cells apparently are able to use as a carbohydrate source some carbohydrate derivative present in the yeast extract that is unavailable to the cytochrome-deficient cells.

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[2] The nutrient medium contains the following per liter: 6 gm bacto yeast extract, 3.5 gm peptone, 2 gm $KH_2PO_4$, 40 gm cerelose, and 30 gm agar, for solid medium.

Matings were made by mixing cells of opposite mating type in a tube of nutrient medium (Lindegren, '49). After 24 hours, the contents of the tubes were diluted and plated on nutrient agar. Diploid colonies were selected by their regular smooth appearance and induced to sporulate, using the technique described by Lindegren ('49). Ascospores were isolated with the aid of a de Fonbrune micromanipulator.

The cultures derived from the isolated ascospores were tested as follows: The mating type was determined by mixing each of the segregant cultures with test strains of both mating types. The mixtures were then observed under the microscope for the presence or absence of the inrregular shapes typical of fusing cells of opposite mating types. Segregant cultures were classified as to fermentative capacity by the ability to produce gas in the insert of a Durham tube containing nutrient medium with the cerelose replaced by the various carbohydrates being tested. Cultures were tested for growth factor requirements by their ability to grow in

Burkholder's ('43) synthetic medium with and without the added growth factor.

The small colonies that appeared on plates seeded with irradiated suspensions were crossed with petites and the mating mixture plated on no-sugar medium to determine whether normal diploids were produced. In order to insure that the normal diploids arose from fusion of two cytochrome-deficient haploid cells, and not from the fusion of a reverted normal haploid cell with the other petite parent, the following procedure was employed. A large inoculum was used to minimize the selection of normals during growth in the mating tube. A portion of the colony was first inoculated into a tube containing 2 ml of liquid medium and allowed to grow for at least a day. Two-tenths milliliter of this suspension was inoculated into each of three tubes containing 2 ml of liquid medium. To the first tube nothing was added; to the second tube a drop of a suspension of petite cells of opposite mating type was added; and to the third a suspension of cells of a second petite culture of opposite mating type. After completion of growth, the supernatant was decanted; and the cells were plated on no-sugar plates. The plates were scored after several days for presence or absence of growth. When few or no colonies appeared on the plate containing the small colony alone and a large number of colonies appeared on the plates containing the matings of the small colonies by petites, the small colonies were classified as mutants. When no colonies appeared on any of the three plates, as was usually the case, the small colonies being tested were classified as petites. In some cases normals appeared on all three plates. Apparently a few normal cells had been picked up in the inoculum taken from the colony, or an early reversion occurred, and since this made it uncertain as to whether the colony was originally mutant or petite, these plates were discarded.

## RESULTS

The rates of killing of a normal, a cytochrome-deficient petite variant of the same normal, and a cytochrome-deficient mutant follow S-shaped curves when plotted on semilogarithmic graph paper as shown in figure 1. It is apparent from the graph that the rate of killing of the two cytochrome-deficient strains does not differ significantly from that of normal yeast.

The number of cytochrome-deficient colonies among the survivors of irradiated normal cells does not follow the same curve, as is shown in figure 2.

Inasmuch as spontaneously occuring petite cells make up 2% of a ''normal'' yeast population, and are killed at the same rate as the normals, as is shown in figure 1, one would expect

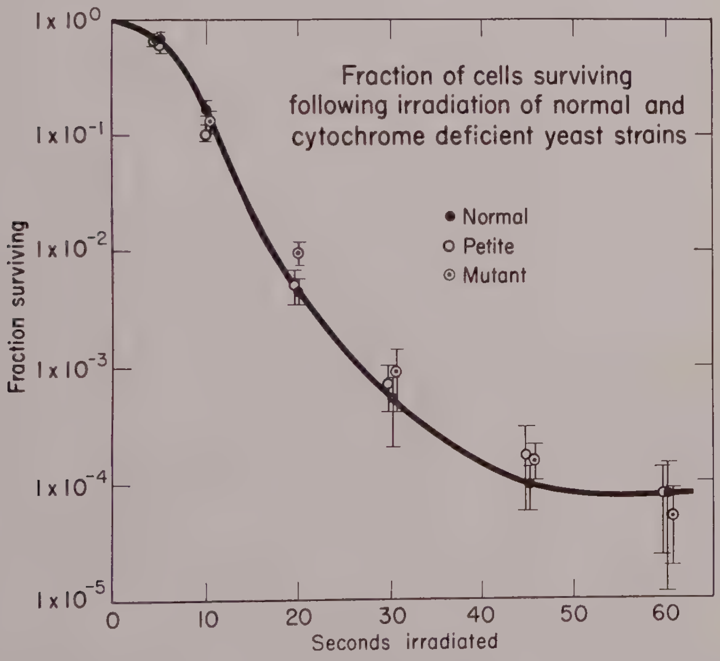

Fig. 1 Fraction of cells surviving following irradiation of normal, mutant, and petite yeast cells. The surviving cells are estimated from a colony count of appropriate dilutions of the irradiated suspensions plated on nutrient agar plates. The points shown in the graph represent averages of from 9 to 18 determinations of the fraction surviving. The range shown on the graph is for the 95% confidence limits of the mean, assuming all the samples to be taken from the same population.

petites to make up 2% of the surviving cells following irradiation. This is shown in the plot of "petites expected" (curve 4, fig. 2). The percentage of cytochrome-deficient colonies obtained, however, far exceeds this prediction, as is shown in the following tabulation:

| | SECONDS IRRADIATED | | | | | | |
|---|---|---|---|---|---|---|---|
| | *0* | *5* | *10* | *20* | *30* | *45* | *60* |
| Percentage of cytochrome-deficient colonies among survivors | 2 | 8 | 20 | 52 | 44 | 21 | 9 |

Furthermore, there is a real increase in the percentage of petites from 2% to 5.1% of the original population following

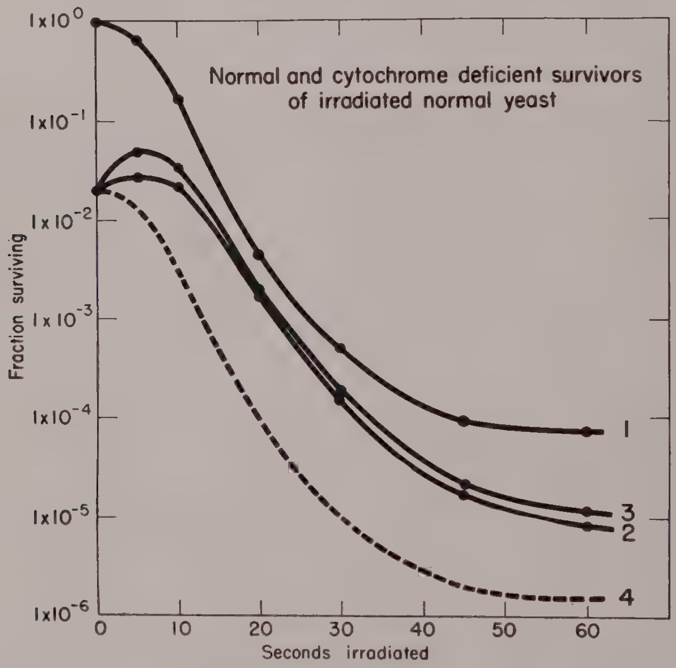

Fig. 2 Fraction of cells surviving irradiation of a normal culture of yeast that are able to form colonies when plated on nutrient agar plates. Curve 1 was obtained from a count of all the colonies, both normal and cytochrome-deficient; curve 2 from a count of small cytochrome-deficient colonies; curve 3 from a count of the cytochrome-deficient colonies plus the mixed colonies containing both normal and cytochrome-deficient cells. The petites expected, shown in curve 4, were calculated from the expected survival of the 2% petites present in the population irradiated.

5 seconds of irradiation and to 3.4% after 10 seconds of irradiation (curve 3, fig. 2). From these two facts, it is clear that small amounts of irradiation, in addition to killing many normal cells, convert some into cytochrome-deficient cells which are shown to be largely petites in experiments described below. The percentages given are for the total petite plus mixed colonies, inasmuch as there appears to be no fundamental difference between these two kinds of colonies. This will be discussed later.

The small colonies appeared to be cytochrome-deficient for several reasons. In the first place, they were small, smooth and white as compared with normal colonies. Secondly, they were shown to be unable to grow on no-sugar medium. Thirdly, when 10 colonies were selected at random and grown in sufficient quantity to observe their absorption spectra, each culture exhibited only the absorption bands characteristic of cytochrome-c at 550 mμ and 520 mμ. These bands are characteristic of petites, as well as of some mutants.

The cytochrome-deficiency is transmitted from one cell generation to the next as is characteristic of a gene mutation. However, if a genic change is postulated, one must also postulate an extraordinarily high mutation rate to explain the high proportion of such cytochrome-deficient colonies among the survivors. Another alternative is to seek an explanation in some form of non-genic but heritable change produced by ultraviolet radiation.

In order to determine whether these cytochrome-deficient colonies were mutant or petite, three of the deficient colonies were selected at random and crossed with a normal culture. These three colonies were among those tested and found to exhibit the absorption bands at 550 and at 520 mμ. The results of the crosses are given in table 1.

Colony 1 appears to be a mutant on the basis of the limited segregation data given above. With one exception the segregants gave the two normal to two cytochrome-deficient segregation expected in a cross between two parents differing in a single gene. The fact that no cytochrome-deficient cultures were found in the progeny of the crosses of colonies 2 and 3 by normals can be most readily accounted for by assuming that they are petites.

In addition to the small colonies, there were some colonies with smooth bases which looked cytochrome-deficient, but with normal-looking papillae covering the surface. Streaking the bases and outgrowths from these mixed colonies on no-sugar plates verified that the bases were cytochrome-deficient and the outgrowths normal. The bases of three such colonies were crossed with a normal and the segregants tested for the ability to grow on no-sugar plates. The results are given in table 2.

TABLE 1

*Segregants of a normal strain crossed with cytochrome-deficient colonies grown from irradiated cells*

| COLONY | NO. ASCI | GROWTH ON NO-SUGAR MEDIUM | | | |
|---|---|---|---|---|---|
| | | Spore 1 | Spore 2 | Spore 3 | Spore 4 |
| I | 3 | + | + | — | — |
| | 1 | + | + | +[1] | — |
| | 3 | + | + | — | 0[2] |
| II | 4 | + | + | + | + |
| | 1 | + | + | + | 0 |
| III | 5 | + | + | + | + |

[1] In all of the cytochrome-deficient segregants a large number of normal outgrowths appeared on the surface of the growth on the stock slants. The extra normal spore clone in this ascus may be due to early occurrence of such an apparent back mutation.

[2] In some asci only three of the four spores germinated.

Two of the three colonies showing outgrowths that were tested appear to be petites, since 44 of the 46 ascospores isolated from the two crosses were normal. The evidence is not sufficient to determine the nature of the two cytochrome-deficient clones which reverted. The base of the third colony however appears to be a mutant, for with one exception, the cytochrome deficiency segregated 2:2. This base was selected for testing because it was the only one of a number of mixed colonies tested that produced normal diploids when crossed with petites. The other two bases were selected at random from among the many that did not produce normals when crossed with petites.

It appears from these limited segregation data that the cytochrome-deficient colonies and the bases of the mixed colonies showing normal outgrowths may be either mutant or petite. The crosses were also heterozygous for mating type, the ability to ferment maltose, and pantothenate independence.

TABLE 2

*Segregants of crosses with normals of the bases of cytochrome-deficient colonies showing outgrowths*

| BASE | NO. ASCI | GROWTH ON NO-SUGAR MEDIUM | | | |
|---|---|---|---|---|---|
| | | Spore 1 | Spore 2 | Spore 3 | Spore 4 |
| I | 2 | + | + | + | + |
| | 2 | + | + | + | 0[1] |
| II | 8 | + | + | + | +[2] |
| III | 9 | + | + | — | — |
| | 1 | + | + | — | 0 |
| | 1 | + | — | — | 0 |
| | 1 | + | + | +[3] | 0 |

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[1] There were a few asci in which only three of the four spores grew.

[2] Two cultures did not grow on no-sugar medium shortly after they were isolated, but some time later were found to be normal.

[3] The extra normal culture in this ascus is very likely due to an early back-mutation to the normal.

These characters segregated regularly showing that there were no meiotic irregularities in the crosses.

It was not practicable to isolate a sufficient number of ascospores from a large enough number of small colony crosses to determine with certainty the classification of the colonies as to mutants or petites. For this reason the colonies were tested in another manner which also provides additional evidence that most of the small colony survivors result from the loss of autonomous non-genic elements from the cells. A number of the small colonies were crossed with petites and the mating mixture plated out on no-sugar plates on which only normal colonies can grow. The petites may be expected to contain the normal gene but to be lacking the non-genic autonomous elements which are also necessary for the production of the complete cytochrome system. On the other hand, the mutants may be expected to have the mutant instead of the normal gene, but to contain the necessary particles. Hence, fusion of the cells of the small colony being tested with petite cells will produce normal diploids if the small colony is mutant; however, if the colony is petite, it will produce only petite diploids when crossed with another petite. It is possible that some of the cells may have lost the non-genic factor and also mutated. If so, since this method will not distinguish between the usual petites which contain the normal gene and such ''petite-mutants'' which contain the mutant gene instead, some of the colonies described as petite may actually be petite-mutants.

It was shown in preliminary experiments that petites crossed with petites produce diploids, but the diploids are not normal. The two petites used in these crosses were shown repeatedly to produce normal diploids when crossed with known recently isolated mutants (e.g. base 3, table 2).

One-hundred-twenty-one cytochrome-deficient colonies did not produce normal diploids when tested by crossing with the two different petite strains. Such colonies apparently are petites (possibly including petite-mutants). Seventeen of these colonies were bases of mixed colonies and the remainder appeared to be homogeneous deficient colonies. Five other deficient colonies produced a large number of normal colonies when crossed with each of the petites, whereas a similar sample of cells from the colony alone was shown to contain no normal cells. This is what would be expected if these 5 colonies were mutants. One of these was the base of a mixed colony, (base 3, table 2). It appears from these data that some of the cytochrome-deficient colonies and the bases of the mixed colonies may be the result of gene mutations, but that the bulk of the colonies are petites.

## DISCUSSION

The evidence to date, (Ephrussi, '49, '51, Raut, '53), indicates that petites are produced by the loss of self dividing non-genic particles from the cells, or at least this assumption is the simplest working hypothesis for the phenomena observed. Therefore, a ready explanation for the production of petites in the experiments under consideration is that such particles are destroyed or inactivated by ultraviolet irradiation.

However, there are several observations which are not readily accounted for by such a simple explanation. In the first place, the bases of the mixed colonies that exhibit rapidly growing outgrowths of normal cells, but do not segregate, seem to be originally petite colonies in which some cells have reverted to normal. Secondly, in spite of the fact that deficient cells are unable to grow on no-sugar medium, when the same number of irradiated cells was streaked on nutrient agar plates with and without sugar, the same number of colonies appeared on both plates. This occurred despite the fact that half the colonies on the plate containing sugar were cytochrome-deficient. It appears from this that some of the cells are so changed by irradiation that they are capable of producing normal or petite colonies depending on the medium on which they grow. This instability seems to be a temporary effect of the irradiation, since it is observed only immediately following irradiation. If this be the case, the irradiation must produce a reversible change in such ''pseudo-petite'' cells which predisposes them to becoming petites. One may envision such a change being due not to a total but to a partial loss of the particles or to a partial or temporary inactivation of the particles or of a cell constituent necessary for particle production. The present evidence does not distinguish between these possibilities.

The increase in the proportion of petites among the survivors following irradiation up to 20 seconds can be accounted for by the conversion of normals to petites by the irradiation.

However, the decrease in the percentage of petites on continued irradiation after 20 seconds needs explanation.

One theory that might account for the shift in the proportion of normals to petites among the survivors is to assume that the pseudo-petites are killed more rapidly than the normals. Another theory that can also account for the shift in the ratio of petites to normals is survival of a very few cells (1) that are radiation resistant both to being killed and to being converted into petites, or (2) that are not hit. The evidence does not distinguish between these two possibilities. However, assuming that a few cells are resistant to being killed or that a few cells are not hit will also account for the leveling off of the rate of kill curves, and this is sufficient to account for the shift in the proportion of petites among the survivors.

The nature of the postulated autonomous particles is not known, although suggestions have been made as to their nature (Mundkur, '53). One may speculate that such particles contain nucleic acids or some other substances which absorb ultraviolet of about 2600 Å and that changes in this material on irradiation are reflected in the physiological characteristics of the cells (e.g. loss of oxidizing capacity by "petites"). That nucleic acids may be involved is strengthened by their universal association with all self-duplicating biological systems so far described.

Results obtained with x-radiation, which will be reported elsewhere, do not show a high rate of conversion of normal cells to petites. This evidence is a consistent with the assumption that the postulated particles may contain nucleic acid.

## SUMMARY

Cytochrome deficiency in yeast resulting in the inability of the cells to consume oxygen does not affect the rate at which cells are killed by ultraviolet light. However, a large proportion of the colonies that appear following irradiation of normal cells are cytochrome-deficient. The cytochrome deficiency is permanent in that it is transmitted from one

cell generation to the next. Some of these deficiencies result from gene mutations, but in other surviving cells the character is not transmitted through a cross, and hence, is not due to a gene mutation. One must conclude that ultraviolet irradiation at 2537 Å causes the disappearance or inactivation of some self-duplicating non-genic elements from the cells. This is substantiated by the fact that in only a few of the deficient survivors tested was it possible to demonstrate the presence of the cytoplasmic factor by transmitting it through crossing to other cells lacking the factor.

## ACKNOWLEDGMENTS

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# PERSISTENT DAILY AND TIDAL RHYTHMS OF $O_2$-CONSUMPTION IN FIDDLER CRABS

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SIX FIGURES

Numerous instances of persistent diurnal rhythms involving various biological processes have been reported in animals (see Welsh, '38, Park, '40, Kleitman, '49, and Webb, '50). To a much more limited extent persisting tidal rhythms have been reported (see Brown, Fingerman, Sandeen, and Webb, '53). Those latter investigators who were studying chromatophore behavior demonstrated the simultaneous possession of both of these types of rhythm by the fiddler crab, *Uca pugnax*. They were able, furthermore, to show that the simultaneous presence of these two rhythms produced, as a result of periodic reinforcement, a persistent semilunar, or 14.8-day, rhythm. The phases of the tidal rhythm were found to be set by the time of the tides in the local habitats of the crabs. Crabs collected from habitats differing in time of low tide by 4 hours were found under constant laboratory conditions to have their tidal rhythms correspondingly 4 hours out of phase with one another, and this difference persisted for the 2 to 4 weeks of observation. The resultant semilunar rhythms were out of phase with one another by a number of days which corresponded with the different tidal phases. The phases of the diurnal rhythm in *Uca pugnax* had been shown earlier (Brown and Webb, '49; Webb, '50) to be readily

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[1] This investigation was supported in part by contracts NONR-09703 and NONR-122803 with the Office of Naval Research, and in part by a grant from the Graduate School of Northwestern University.

reset by artificial light to dark or dark to light changes at sensitive periods in the diurnal cycle and thereafter under constant conditions bear relationships other than the normal to the natural day-night phases. When the diurnal rhythm was abruptly shifted by light changes, the phases of the tidal rhythm shifted in the same direction and to the same extent.

Brown, Fingerman, Sandeen, and Webb ('53) postulated that such endogenous rhythmic mechanisms as those regulating the chromatophores might well be those directly responsible for regulating much of the known periodic behavior of organisms whether of tidal, diurnal or lunar frequency, e.g. feeding and reproduction. Strongly supporting this view was the demonstration by Brown and Webb ('48) that the frequency of the diurnal rhythm was independent of temperature over the wide range of 6 to 26°C. This highly specialized property of the endogenous rhythm is obviously an essential one if the rhythm is to play such a role in these poikilotherms. Further observations are suggesting that the frequency of the tidal rhythm is also temperature-independent over a broad temperature range.

The chromatophores and color changes in Uca are directly regulated by endocrines. Many investigators have suspected that the same hormones which regulated the chromatophores also influence other more basic physiological processes. An influence on heart rate, for example, was strongly suggested by Scudamore ('41) and it is known that heart rate typically reflects organismic $O_2$ demands. On the other hand, Gompel ('37) has reported persistent tidal rhythms with about 12.4-hour cycles in a number of species of marine animals, including both invertebrates and vertebrates.

It was the intention in this study to learn to what extent the rate of $O_2$-consumption in the fiddler crab might be regulated by the diurnally and tidally rhythmic centers, and therefore to what extent the great variations in $O_2$-consumption normally observed in such organisms might be orderly and predictable in terms of these endogenous rhythms.

## MATERIALS AND METHODS

The animals used in the experiments to be described were male Uca pugnax and Uca pugilator collected at Chapoquoit, near Woods Hole, Massachusetts. For the first series of experiments, U. pugnax were collected at about three-day intervals between July 18 and 30, 1953. For the second series, crabs of the same species were collected at similar intervals between August 2nd and 14th. The crabs were usually placed in the respirometers within a few hours of the time they were brought into the laboratory. For the third, fourth, and fifth series, a large supply of U. pugnax and pugilator were collected on August 23rd and placed in large white-enamelled dish-pans at a constant illumination of about 3 ft. c. At 2-to 3-day intervals between August 23rd and September 6th specimens of U. pugnax were taken from the pans and placed in respirometers. At similar intervals specimens of normal U. pugilator were similarly placed in respirometers, as were also a few specimens of U. pugilator from which eyestalks had been amputated a day or two previously. No individual crabs were kept in the respirometer chambers for more than about 3 days, and after such a period of measurement of their $O_2$-consumption they were always discarded.

The respirometers which were used were of the type described by Brown ('54). These permitted one to obtain a continuous record of the variations in rate of $O_2$-consumption in undisturbed animals under constant conditions over a period of 3 or 4 days. The method was in essence as follows. The animal was placed in a diver consisting of a 50 ml soxhlet distilling flask containing the animal together with vials holding $CO_2$ and ammonia absorbents. A rubber stopper in the flask was penetrated by a 27 gauge hypodermic needle leading into a plastic (Saran) collapsible sack sealed to the upper end of the rubber stopper. The sack was filled with oxygen. The whole diver was weighted just enough to cause it to submerge completely and was suspended by thread to an ink-recording spring scale producing a continuous record of the weight of the diver on a slow-moving strip of paper drawn over a drum at a rate of about 2 cm an hour. It is obvious that in such a system, oxygen will flow from the plastic sack into the flask as fast as $O_2$ is consumed by the organism and that the diver will increase in weight by 1 gm for each milliliter of $O_2$ consumed. An excess of $O_2$ was always present in the sack. The sacks of the divers each had a capacity of about 40 ml, enough to last an animal about a week at their typical rate of utilization.

For the experiments with Uca pugnax, two constant temperature baths were used, one with 5 and the other with 6 respirometers. One of the baths was maintained at 24.6° C. and the other at 25.1° C. At the beginning of the 15-day run for each series, one crab was placed in each of 4 respirometers in one bath and one in each of 5 in the other. The remaining respirometer in each bath was run as a blank control, set up precisely as the others except for the absence of a crab. At two- to three-day intervals, the respirometers of each bath were dismantled and set up afresh with new animals. The respirometers in the two baths were always set up a number of hours apart in order that there would be a smoothly overlapping record for the crabs as a whole over the 15-day period.

The third, fourth and fifth series were run simultaneously using the same baths that had been used for the first two series. A total of 10 respirometers were now used in each bath. The additional 9 respirometers were given over to U. pugilator, 4 or 5 to the normal and the remainder to the eyestalkless animals.

The baths containing the respirometers were kept during all 5 series in an inside room in which the illumination was kept constant. The illumination at the surface of the water of the galvanized-iron water baths was about 3 ft. c.

From the records that were obtained from the respirometers, it was readily possible to determine the average $O_2$-consumption for each hour for the several animals of each series over the whole 15-day or half-lunar period.

## RESULTS

The first continuous 15-day recording of $O_2$-consumption was obtained between July 18th and August 1st, inclusive, using Uca pugnax. In table 1 are to be found the hourly average rates of $O_2$-consumption for 3 to 9 crabs during the course of this semilunar period. Since these values are all averages, it is obvious that the total range of values for individual animals during this period was substantially greater. The results in table 1 are expressed in ml/kg/hr., still uncorrected, however, for the average increase in weight of blank control respirometers.

If one assumes that the animals have persistent diurnal and tidal rhythms of $O_2$-consumption comparable to the rhythms of color changes observed in these animals, then at least some of the variations in rate of $O_2$-consumption would be capable of resolution into cycles of 24-hour and 12.4-hour lengths. It is possible to randomize the effects of any 12.4-hour tidal rhythm which might be present by obtaining the average rate of respiration for each hour of the day for the whole 15-day period. In the course of a period of such length, each of two tidal cycles whose phases are moving across the daily period at a rate of about 50 minutes a day, would have just scanned half of a daily period once and all phases of a single tidal cycle would have been represented in each of the hourly averages of the day. The results obtained from such an average are shown in figure 1 A and clearly demonstrate that there is a persistent diurnal rhythm characterized by a maximum rate of $O_2$-consumption about 8 to 9 A.M., then a rapid reduction in rate to a minimum about noon followed by an irregular increase to a second maximum about 10 to 11 in the evening with a second minimum occurring about midnight. These values in figure 1 have been corrected for non-respiratory increase in respirometer weight to give the actual average hourly rates of $O_2$-consumption.

A second series was run during the 15-day period from August 2nd to 16th using the same species collected from the

TABLE 1

*$O_2$-consumption in Uca pugnax in ml/kg/hr.*

| | A.M. | | | | | | | | | | | | P.M. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *July* 18 | 15 | 52 | 50 | 45 | 45 | 55 | 65 | 63 | 67 | 23 | 10 | 25 | 10 | 25 | 20 | 35 | 27 | 67 | 17 | 15 | 30 | 32 | 25 | 8 |
| 19 | 40 | 15 | 35 | 25 | 52 | 53 | 58 | 37 | 73 | 17 | 20 | 26 | 20 | 17 | 26 | 57 | 54 | 36 | 34 | 42 | 60 | 40 | 50 | 37 |
| 20 | 45 | 60 | 75 | 56 | 56 | 81 | 82 | 60 | 70 | 81 | 32 | 32 | 31 | 46 | 36 | 56 | 25 | 35 | 30 | 82 | 46 | 56 | 52 | 68 |
| 21 | 30 | 44 | 34 | 26 | 37 | 42 | 59 | 37 | 70 | 45 | 87 | 27 | 45 | 40 | 46 | 19 | 39 | 50 | 19 | 20 | 52 | 52 | 41 | 40 |
| 22 | 51 | 36 | 20 | 17 | 37 | 44 | 36 | 45 | 80 | 13 | 35 | 46 | 34 | 28 | 26 | 17 | 36 | 22 | 39 | 21 | 62 | 64 | 44 | 46 |
| 23 | 44 | 34 | 70 | 26 | 27 | 39 | 30 | 25 | 56 | 13 | 52 | 29 | 31 | 26 | 27 | 15 | 9 | 10 | 13 | 25 | 27 | 39 | 22 | 110 |
| 24 | 48 | 37 | 51 | 37 | 95 | 67 | 52 | 35 | 62 | 50 | 46 | 32 | 38 | 32 | 20 | 15 | 17 | 35 | 32 | 32 | 38 | 58 | 37 | 40 |
| 25 | 80 | 70 | 61 | 56 | 46 | 40 | 66 | 41 | 50 | .. | .. | .. | .. | 50 | 67 | 38 | 35 | 46 | 53 | 40 | 61 | 90 | 55 | 67 |
| 26 | 22 | 43 | 51 | 38 | 35 | 27 | 42 | 25 | 44 | 39 | 18 | 4 | 3 | 13 | 9 | 12 | 38 | 20 | 18 | 8 | 8 | 22 | 20 | 16 |
| 27 | 8 | 20 | 26 | 70 | 30 | 22 | 30 | 25 | 35 | 2 | 25 | 40 | 23 | 30 | 12 | 12 | 43 | 26 | 59 | 43 | 48 | 55 | 30 | 110 |
| 28 | 92 | 90 | 69 | 117 | 90 | 80 | 68 | 27 | 53 | 45 | 43 | 60 | 71 | 76 | 74 | 106 | 60 | 63 | 86 | 55 | 36 | 40 | 170 | 14 |
| 29 | 68 | 70 | 38 | 64 | 83 | 58 | 98 | 52 | 44 | 30 | 47 | 20 | 25 | 71 | 72 | 85 | 30 | 35 | 42 | 58 | 62 | 60 | 42 | 72 |
| 30 | 28 | 46 | 57 | 82 | 70 | 67 | 70 | 45 | 102 | 22 | 49 | 32 | 38 | 55 | 65 | 57 | 68 | 39 | 56 | 37 | 45 | 59 | 114 | 30 |
| 31 | 42 | 45 | 49 | 55 | 72 | 68 | 60 | 50 | 65 | 36 | 20 | 18 | 16 | 19 | 60 | 50 | 30 | 30 | 80 | 78 | 80 | 70 | 124 | 50 |
| *Aug.* 1 | 46 | 51 | 60 | 66 | 58 | 58 | 52 | 52 | 57 | 48 | 50 | 23 | 21 | 44 | 45 | 35 | 63 | 48 | 40 | 44 | 35 | 61 | 36 | 46 |

same habitat and treated in essentially the same manner. A similar treatment of the uncorrected hourly values which were obtained, table 2, resulted in the demonstration of the same general form of a persistent diurnal rhythm (fig. 1 B — corrected values), but with very slight differences in the times of day of the maxima and minima. During the progress of this series, it became evident that the unlubricated bearings in the recording spring scales of the respirometers were becoming somewhat corroded and that the consequent increase in the frictional resistance of the recording system might be at least

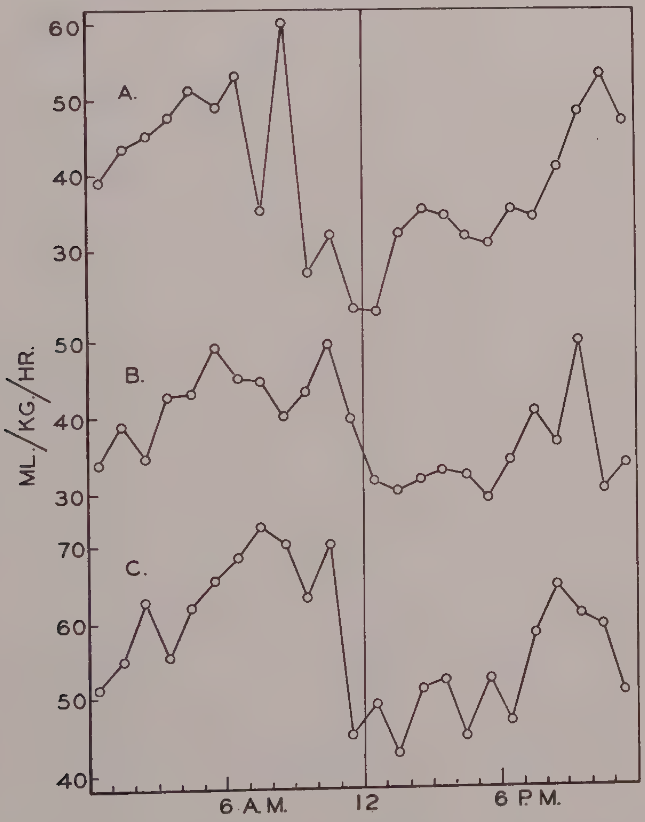

Fig. 1 The diurnal rhythm of $O_2$-consumption in *Uca pugnax* obtained by randomizing the tidal rhythm for the semilunar periods (A) July 18th–Augst 1st, (B) August 2nd–August 16th, and (C) August 22nd–September 5th.

## TABLE 2

*$O_2$-consumption in Uca pugnax in ml/kg/hr.*

| | A.M. | | | | | | | | | | | | P.M. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *Aug.* 2 | 4 | 64 | 30 | 21 | 39 | 61 | 50 | 44 | 30 | 28 | 49 | 50 | 20 | 40 | 22 | 38 | 34 | 38 | 56 | 74 | 67 | 52 | 46 | 58 |
| 3 | 62 | 86 | 30 | 80 | 74 | 64 | 42 | 52 | 26 | 36 | 40 | 38 | 68 | 14 | 30 | 46 | 18 | 56 | 58 | 58 | 32 | 34 | 48 | 30 |
| 4 | 42 | 34 | 56 | 52 | 48 | 66 | 26 | 58 | 24 | 82 | 28 | 16 | 32 | 26 | 14 | .. | .. | 14 | 44 | 30 | 30 | 62 | 56 | 26 |
| 5 | 18 | 24 | 22 | 90 | 34 | 56 | 72 | 58 | 50 | 52 | 56 | 58 | 42 | 72 | 78 | 40 | 92 | 84 | 90 | 64 | 72 | 134 | 66 | 84 |
| 6 | 72 | 72 | 74 | 36 | 68 | 48 | 68 | 92 | 84 | 60 | 118 | 64 | 74 | 54 | 48 | 42 | 44 | 19 | 64 | 60 | 13 | 45 | 29 | 58 |
| 7 | 49 | 59 | 44 | 28 | 45 | 30 | 63 | 33 | 68 | 39 | 59 | 26 | 35 | 31 | 56 | 68 | 17 | 18 | 18 | 27 | 42 | 56 | 22 | 45 |
| 8 | 42 | 42 | 27 | 29 | 33 | 29 | 37 | 69 | 59 | 58 | 33 | 43 | 28 | 38 | 36 | 47 | 23 | 39 | 31 | 47 | 29 | 58 | 41 | 32 |
| 9 | 65 | 35 | 40 | 48 | 52 | 20 | 34 | 24 | 66 | 36 | 78 | 95 | 12 | 26 | 8 | 25 | 31 | 16 | 28 | 36 | 53 | 46 | 35 | 40 |
| 10 | 30 | 40 | 43 | 51 | 49 | 31 | 46 | 36 | 29 | 48 | 44 | 49 | 41 | 61 | 36 | 29 | 29 | 29 | 20 | 46 | 43 | 91 | 44 | 38 |
| 11 | 35 | 38 | 44 | 40 | 49 | 49 | 34 | 39 | 35 | 41 | 40 | 35 | 35 | 28 | 45 | 40 | 43 | 25 | 40 | 35 | 18 | 45 | 16 | 32 |
| 12 | 57 | 43 | 47 | 62 | 44 | 68 | 48 | 33 | 28 | 48 | 65 | 38 | 29 | 39 | 32 | 36 | 42 | 39 | 30 | 29 | 54 | 33 | 33 | 41 |
| 13 | 59 | 29 | 40 | 44 | 47 | 74 | 43 | 28 | 31 | 29 | 34 | 31 | 33 | 21 | 36 | 30 | 29 | 32 | 28 | 95 | 48 | 21 | 24 | 26 |
| 14 | 19 | 29 | 36 | 25 | 33 | 53 | 38 | 30 | 26 | 58 | 85 | 13 | 24 | 36 | 30 | 26 | 46 | 13 | 26 | 24 | 31 | 24 | 06 | 01 |
| 15 | 03 | 06 | 11 | 50 | 47 | 84 | 66 | 84 | 53 | 34 | 42 | 77 | 40 | 30 | 49 | 44 | 50 | 69 | 24 | 27 | 33 | 38 | 31 | 46 |
| 16 | 36 | 55 | 56 | 69 | 66 | 59 | 56 | 44 | 46 | 59 | 33 | 13 | 29 | 28 | 40 | 26 | 33 | 34 | 39 | 30 | 56 | 76 | 40 | 38 |

partially responsible for the slightly reduced amplitude of the diurnal rhythm which was observed. Therefore, the recording system was completely reconstructed; new, improved, and lubricated bearings in the scales were substituted for the previous ones and on August 22nd a third 15-day series was commenced, again using Uca pugnax collected at Chapoquoit. The uncorrected hourly results of this study are seen in table 3 and the form of the diurnal rhythm after randomizing any possible tidal cycles is illustrated with corrected values in figure 1 C. Again the same form of the persistent diurnal rhythm was evident, now of greater amplitude and perhaps agreeing a little more closely with the times of the maxima and minima of series 2 than of series 1.

Despite the fact that in series 3 all the animals employed in the experiments had been kept under constant conditions of illumination and under essentially constant conditions of temperature for the whole 15 days, the general form and temporal relationship of the phases of the observed daily rhythm of $O_2$-consumption were not significantly different from those found in series 1 and 2 for which the animals had been brought in from the field about the time they were to start their two- to three-day sojourn in the respirometers.

In series 1, the average hourly respiratory rates for the semilunar period was 39.8 ml/kg/hr. and ranged from a noon low of about 22 ml/kg/hr. to mid-morning high of about 60 ml/kg/hr., or an increase of about 2.7 times. In series 2, the overall average was 37.3 ml/kg/hr. ranging from an average daily low of 28.5 to a high of about 50. This amounts to an increase by a factor of only 1.7. In series 3 the average was higher, 57.8, and the range in the daily cycle varied from a noon low of 42.5 to a morning high of 72.5, or an increase from lowest to highest of 1.7 times, just as in series 2.

The reproducibility of the form of the daily variation in rate of $O_2$-consumption over the three 15-day periods leaves no doubt as to the reality of a precise endogenous rhythmic mechanism regulating physiological processes responsible for these differences in the rates of gaseous exchange in Uca pugnax.

A study of the variations in rates of $O_2$-consumption for the individual days in a 15-day period, on the other hand, showed the general form of the daily variations to differ from day to day. On no day did the pattern of variation very closely simulate the diurnal one which has been discussed earlier. There was, however, a somewhat systematic change in pattern from day to day which appeared capable of interpretation in terms of there being generally a higher rate of $O_2$-consumption near the times of low tide, though this seemed not to be invariable. At all events, in the systematic alteration in form of the daily pattern of $O_2$-consumption, the first and last days of a 15-day period of recording appeared to be more closely similar to one another in general form than to any other day in the series. The latter is what one would expect were there a persisting tidal rhythm, since at about 15-day intervals, the times of two low tides would come to repeat any given temporal relations to the daily cycle.

The next step in the analysis of the data was to attempt to randomize the diurnal rhythm while simultaneously synchronizing the cycles of whatever tidal rhythm might be present. This was done in the following manner: The hourly data of the second day in a series was placed directly below the data of the first day as in tables 1 through 3. The data of the third day, however, was placed beneath that of the second day but now displaced to the left by one hour so that, for example, the 11:30 P.M. value of day 3 lay beneath the 10:30 P.M. value for day 2. The 12:30 A.M. value of day 3 was then placed beneath the 11:30 P.M. value of day 2. The data of day 4 was displaced to the left by still another hour, and now the 12:30 and 1:30 A.M. values of day 4 were carried over to lie under the 10:30 and 11:30 P.M. values, respectively, of day 3. The data of the 5th and 6th days were similarly shifted to the left by an hour each, and the data for the early hours of these two days correspondingly moved to occupy positions under evening values of the first two days. It is now obvious that

TABLE 3

*$O_2$-consumption in Uca pugnax in ml/kg/hr.*

| | A.M. | | | | | | | | | | | | P.M. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *Aug.* 22 | 85 | 76 | 71 | 63 | 64 | 92 | 103 | 141 | 93 | 75 | 82 | 52 | 36 | 40 | 25 | 97 | 47 | 43 | 55 | 15 | 20 | 74 | 79 | 77 |
| 23 | 97 | 78 | 65 | 57 | 66 | 61 | 60 | 97 | 90 | 70 | 70 | 45 | 69 | 58 | 52 | 35 | 48 | 27 | 40 | 36 | 53 | 115 | 42 | 46 |
| 24 | 42 | 39 | 49 | 42 | 55 | 60 | 45 | 62 | 95 | 94 | 78 | 70 | 44 | 43 | 50 | 62 | 62 | 71 | 52 | 112 | 185 | 58 | 83 | 106 |
| 25 | 107 | 126 | 124 | 58 | 80 | 106 | 85 | 73 | 100 | 132 | 121 | 104 | 91 | 76 | 76 | 68 | 62 | 62 | 58 | 91 | 35 | 72 | 35 | 54 |
| 26 | 51 | 96 | 97 | 73 | 61 | 86 | 46 | 68 | 58 | 38 | 62 | 58 | 122 | 61 | 43 | 58 | 70 | 46 | 70 | 78 | 52 | 85 | 96 | 55 |
| 27 | 82 | 140 | 99 | 106 | 105 | 90 | 93 | 63 | 113 | 64 | 44 | 66 | 67 | 52 | 97 | 54 | 64 | 82 | 64 | 158 | 151 | 44 | 131 | 73 |
| 28 | 54 | 80 | 92 | 76 | 80 | 59 | 95 | 80 | 73 | 77 | 64 | 56 | 44 | 52 | 70 | 87 | 45 | 44 | 71 | 70 | 55 | 18 | 34 | 35 |
| 29 | 35 | 35 | 44 | 41 | 55 | 64 | 67 | 82 | 58 | 59 | 43 | 21 | 44 | 68 | 55 | 50 | 92 | 158 | 64 | 56 | 40 | 44 | 71 | 36 |
| 30 | 32 | 45 | 64 | 65 | 93 | 61 | 101 | 71 | 38 | 65 | 101 | 35 | 67 | 35 | 120 | 86 | 79 | 77 | 37 | 32 | 116 | 81 | 30 | 34 |
| 31 | 23 | 38 | 43 | 69 | 87 | 87 | 58 | 65 | 48 | 37 | 78 | 32 | 26 | 49 | 35 | 39 | 63 | 79 | 38 | 36 | 36 | 137 | 70 | 65 |
| *Sept.* 1 | 89 | 49 | 63 | 50 | 78 | 80 | 101 | 70 | 78 | 103 | 62 | 75 | 58 | 67 | 29 | 43 | 26 | 41 | 57 | 50 | 77 | 50 | 60 | 57 |
| 2 | 68 | 69 | 56 | 50 | 64 | 58 | 78 | 66 | 95 | 51 | 45 | 38 | 50 | 29 | 48 | 24 | 14 | 45 | 46 | 66 | 48 | 69 | 71 | 67 |
| 3 | 62 | 60 | 85 | 83 | 61 | 85 | 83 | 121 | 119 | 89 | 121 | 52 | 70 | 83 | 108 | 129 | 81 | 61 | 76 | 77 | 112 | 57 | 83 | 71 |
| 4 | 57 | 61 | 87 | 93 | 91 | 55 | 94 | 95 | 69 | 82 | 53 | 92 | 60 | 40 | 73 | 59 | 38 | 45 | 72 | 92 | 91 | 194 | 109 | 88 |
| 5 | 43 | 79 | 52 | 59 | 52 | 93 | 72 | 73 | 81 | 65 | 79 | 32 | 47 | 41 | 43 | 46 | 39 | 61 | 57 | 61 | 54 | 69 | 54 | 51 |

the daily cycles have been gradually moved over one another by 5 hours in 6 days, or at an average rate of 50 minutes a day. Since the tidal cycles normally move forward over the daily cycle at about 50 minutes a day, any endogenous rhythm of tidal frequency has had its cycles kept rather closely in phase with one another in the vertical columns of the rearranged data of the successive days. By continuing with the data of the remaining days, repeating this pattern of shifting until all 15 days of the data are used, it is quite clear that a diurnal cycle has gradually moved half way across the 24 vertical columns. If one now uses again the data of the first 14 days but now continues to shift the daily periods to the left beneath one another at the same rate of 5 hours of shift in each 6 days, then it is readily apparent that one daily cycle has completely scanned the 24 vertical columns of data a single time, while in these vertical columns any existing tidal rhythm on the successive days has been kept closely in phase.

An analysis of the data in the manner just described to randomize the endogenous diurnal rhythm and expose the character of any endogenous tidal rhythm present indicated very clearly that a persistent tidal rhythm of $O_2$-consumption was present in the crabs used in all three experimental series. The detailed form of the tidal cycles varied among the three series and seemed to suggest that the form was not a simple one. In all three, however, the time of maximum $O_2$-consumption occurred about an hour or two before the time of low tide in the native habitat of the crabs, and the time of minimum $O_2$-consumption was in every instance just about 6 hours following the time of a low tide, or close to the time of high tide.

The results obtained in the first series is illustrated in figure 2 A. In this figure, the time of low tide on the 24-hour abscissal scale may be determined by the time of low tides on the day when the series began. This was between 6:30 and 7:00 A.M. and P.M. D.S.T. in the first series and about 7:00 A.M. and P.M. D.S.T. in the second. In the third series, in order to have the maxima and minima of the tidal cycles lying at approximately the same relative positions along the abscissa, the data were simply transposed, which is quite legitimate since the abscissal values do not describe any fixed time in a daily cycle. In addition to exhibiting approximately two cycles a day as expected, these tidal cycles in every instance give some indication of being about the expected 12.4 hours in length. In the first series, both the maxima and minima appear to be somewhat more than 12 hours apart; the same situation prevails in figure 2 B taken from series 2. For series 3, plotted in figure 2 C, the minima appear to be close to 12

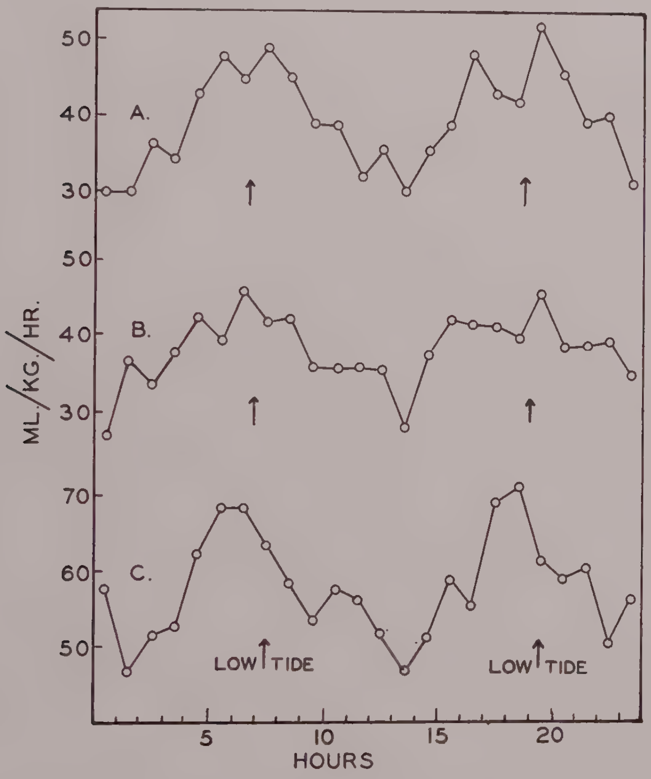

Fig. 2 The tidal rhythm of $O_2$-consumption in Uca pugnax obtained by randomizing the diurnal rhythm for the semilunar periods (A) July 18th–August 1st, (B) August 2nd–August 16th, and (C) August 22nd–September 5th.

hours apart, but the maxima show again clear indication of being something greater than 12.

The average range of the tidally rhythmic variation in $O_2$-consumption in series 1 is seen to be from 30 to 52 ml /kg/hr.; in series 2 it ranges from 26.5 to 46 ml/kg/hr., and in series 3 from 46.5 to 71. From lowest to highest, values for the cycles of the three series are, respectively, 74%, 73%, and 52% increases.

It is evident from the preceding analyses that there exists within U. pugnax, endogenous rhythms having frequencies that at least closely approximate diurnal and tidal ones. Each of these rhythms was responsible for orderly changes in rate of oxygen consumption amounting to a 25 to 50% variation about the mean during a semilunar period. It was not possible to predict how these two rhythmic effects would supplement or antagonize one another as their phase relations altered with respect to one another. Furthermore, it was not possible from the previous type of analysis to determine to what extent these two types of cycles might actually differ in lengths from the natural environmental cycles. If these two cycles were the expected 24 hours and 12.4 hours, then they would be expected to repeat any given phase relationships to one another every 15 days. Within the 15-day period, there would be a systematic movement of tidal cycles over the diurnal at the rate of about 50 minutes a day.

The average forms of the daily curves of variations in $O_2$-consumption for each of the 15 days in a semilunar period are illustrated in figure 3. These were obtained in the following manner. The daily variations for each of the three series that were run for U. pugnax were smoothed by using as the rate for each hour of the day, an average of three values, that hour and the preceding and following one. The daily variations for the three 15-day series were then aver-

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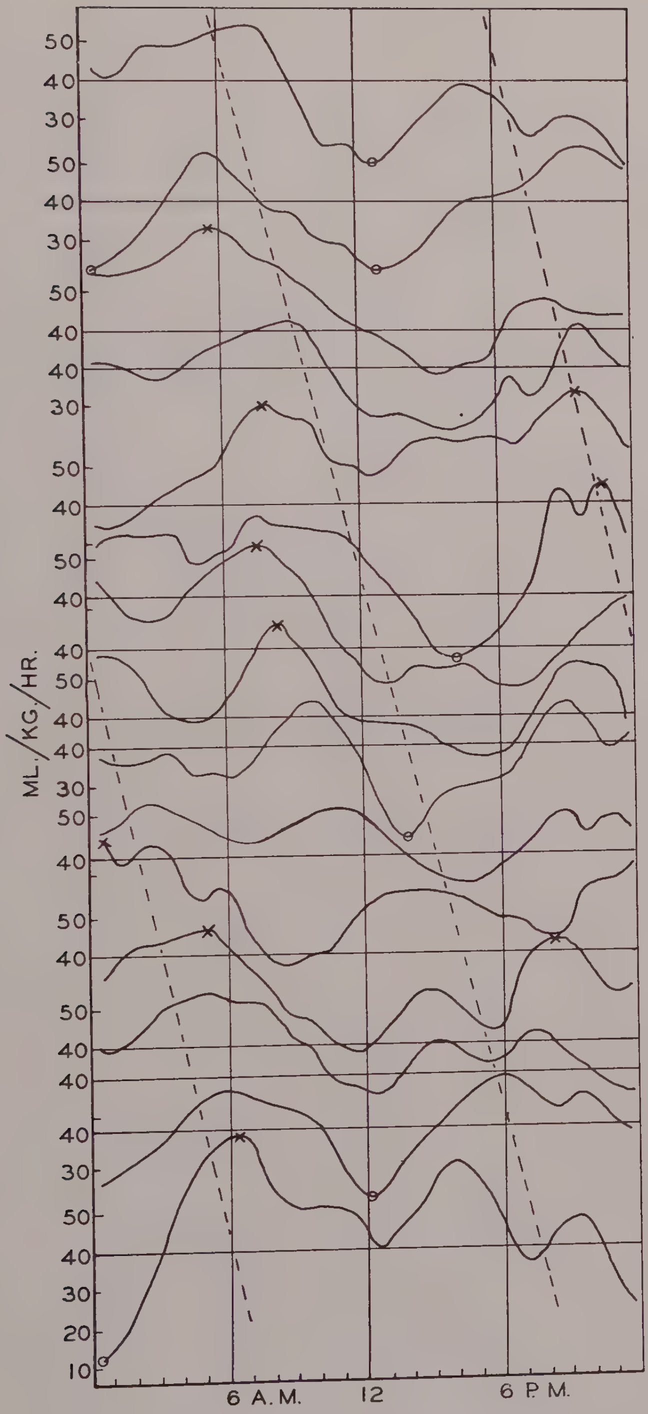
Fig. 3 The average daily variation in the rate of $O_2$-consumption for the three semilunar cycles during which the records were obtained for *Uca pugnax*. The oblique broken lines indicate the approximate time of a low tide on the various days in the 15-day cycle. X's indicate times of high (65–73) and O's, times of low (12–22) $O_2$-consumption.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20
10
ML./KG./HR.
6 A.M.
12
6 P.M.

Figure 3

aged together to form composite daily curves after matching those days in each of the three series when the relationship of the tidal rhythm to the diurnal was most closely similar. Since 4 days of data were available for the first and last days in the three semilunar series obtained, only two days were averaged to characterize the first day in the series, and the other two were used to provide the data for the last or 15th day in the series. This was done only to render more significant any finding that the general pattern of the daily variations in rates of $O_2$-consumption repeat themselves about every 15 days.

A study of figure 3 reveals a number of interesting points. The first and last days in the series show, as expected, an extraordinary similarity in the times of maxima and minima during the day. To be a maximally precise tidal rhythm of 12.4 hr. cycles, these daily curves should repeat their form, on an average, every 14.8 days. In this series, it would appear that the best match of the daily curve of the first day in the series actually does lie between the 14th and the 15th day, rather than on the 15th. No other curve of daily variation resembles as closely the curves of the first and last days, nor do any other two closely resemble one another. There appears, therefore, to be a rhythm having cycles of quite accurate 12.4-hour length.

It has earlier been shown that the diurnal rhythm has its frequency independent of temperature over at least the range, 6° to 26°C., in another species of fiddler crab. These results suggest strongly that the tidal rhythm frequency is also independent of temperature over a rather broad range of temperatures, since these crabs were placed in constant low illumination at 25°C., a temperature definitely higher than the average temperature in the environment from which they had been taken. Nonetheless, they had precisely the expected rhythm frequency.

Certain other things are also evident from figure 3. The highest rates of $O_2$-consumption in the lunar cycle (65 to 73 ml/kg/hr.), which are indicated by the X's on the curves, almost always occur at a time near when there would be expected to be a supplementation of a diurnal peak by a tidal peak. They are not found between 9 in the morning and 8 in the evening. The lowest rates (12 to 22 ml/kg/hr.), which are indicated by solid circles, occur in the overwhelming majority of the cases at times when minimal supplementation by the tidal rhythm occurs near the time of either the noon or midnight diurnal low.

The daily variations depicted in figure 3 represent greatly smoothed daily relationships not only as a consequence of averaging of the several individuals, but also from the averaging together of overlapping periods during the day and the averaging of the three 15-day series. Despite this, it is seen that from the lowest average rate of $O_2$-consumption (12 ml/kg/hr.) to the highest (73 ml/kg/hr.) of the 15-day cycle involves about a 6-fold increase.

### *Uca pugilator*

The same kind of an analysis for rhythms of $O_2$-consumption as were made for the Uca pugnax were also made for normal and eyestalkless U. pugilator. The eyestalkless ones in each instance had had their stalks removed about a day before their two-to three-day recording of respiratory rate. The experiments for these were performed from August 22nd to September 5th, inclusive. All the crabs for these two series were brought into the laboratory simultaneously on August 21st and placed in a constant illumination of about 5 ft. c. in white dish-pans. Four to 6 animals in each condition, normal and eyestalkless, were studied at a time. Normal ones were almost constantly in the respirometers during this 15-day period. Unfortunately, in the case of the eyestalkless ones, two complete days of records were lacking in the 15-day series.

The data for the normal U. pugilator are given in table 4 and for the eyestalkless ones in table 5.

The normal animals exhibited a persistent diurnal rhythm (figure 4 A) which resembled rather closely that shown earlier for U. pugnax. In pugilator, however, the morning maximum

TABLE 4

*$O_2$-consumption in Uca pugilator in ml/kg/hr.*

| | A.M. | | | | | | | | | | | | P.M. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *Aug.* 22 | 156 | 149 | 132 | 135 | 107 | 134 | 165 | 109 | 183 | 90 | 146 | 160 | 18 | 61 | 47 | 99 | 35 | 58 | 106 | 73 | 117 | 144 | 134 | 195 |
| 23 | 157 | 147 | 174 | 86 | 76 | 79 | 85 | 134 | 95 | 65 | 69 | 60 | 55 | 91 | 53 | 36 | 67 | 50 | 40 | 87 | 98 | 37 | 29 | 26 |
| 24 | 26 | 44 | 39 | 35 | 43 | 33 | 34 | 47 | 43 | 94 | 69 | 101 | 69 | 106 | 77 | 88 | 88 | 68 | 37 | 154 | 79 | 66 | 83 | 126 |
| 25 | 91 | 169 | 117 | 108 | 141 | 137 | 142 | 100 | 123 | 86 | 121 | 194 | 121 | 110 | 106 | 73 | 85 | 81 | 68 | 72 | 50 | 9 | 9 | 30 |
| 26 | 56 | 112 | 92 | 92 | 74 | 81 | 40 | 35 | 18 | 65 | 44 | 41 | 110 | 88 | 103 | 111 | 111 | 127 | 141 | 137 | 131 | 127 | 132 | 143 |
| 27 | 161 | 137 | 159 | 150 | 178 | 178 | 167 | 149 | 81 | 37 | 58 | 35 | 62 | 47 | 97 | 96 | 84 | 107 | 102 | 207 | 145 | 105 | 97 | 94 |
| 28 | 105 | 115 | 124 | 111 | 135 | 92 | 118 | 103 | 77 | 64 | 47 | 71 | 73 | 87 | 97 | 180 | 82 | 126 | 120 | 113 | 126 | 27 | 62 | 89 |
| 29 | 45 | 55 | 94 | 91 | 142 | 88 | 94 | 111 | 94 | 95 | 53 | 52 | 58 | 47 | 117 | 115 | 102 | 177 | 86 | 51 | 67 | 56 | 69 | 76 |
| 30 | 67 | 93 | 99 | 112 | 102 | 99 | 97 | 139 | 115 | 109 | 223 | 151 | 202 | 29 | 122 | 94 | 124 | 115 | 176 | 199 | 291 | 121 | 178 | 130 |
| 31 | 207 | 166 | 192 | 135 | 165 | 164 | 185 | 174 | 144 | 115 | 84 | 95 | 69 | 39 | 109 | 106 | 118 | 122 | 127 | 128 | 100 | 159 | 151 | 96 |
| *Sept.* 1 | 96 | 84 | 139 | 147 | 139 | 135 | 172 | 149 | 86 | 176 | 53 | 187 | 180 | 143 | 157 | 173 | 121 | 113 | 204 | 146 | 146 | 172 | 126 | 148 |
| 2 | 158 | 145 | 124 | 161 | 140 | 117 | 123 | 155 | 152 | 127 | 99 | 96 | 112 | 122 | 102 | 69 | 94 | 101 | 138 | 112 | 143 | 105 | 110 | 102 |
| 3 | 128 | 123 | 111 | 115 | 114 | 173 | 178 | 172 | 158 | 181 | 141 | 139 | 120 | 99 | 104 | 85 | 76 | 103 | 79 | 96 | 139 | 154 | 59 | 39 |
| 4 | 56 | 88 | 126 | 131 | 95 | 172 | 175 | 133 | 148 | 148 | 123 | 98 | 107 | 138 | 124 | 91 | 79 | 141 | 94 | 88 | 120 | 167 | 95 | 103 |
| 5 | 126 | 112 | 126 | 91 | 143 | 165 | 137 | 163 | 137 | 115 | 167 | 85 | 84 | 97 | 79 | 79 | 126 | 104 | 99 | 103 | 123 | 224 | 84 | 79 |

TABLE 5

*$O_2$-consumption in eyestalkless Uca pugilator in ml/kg/hr.*

| | A.M. | | | | | | | | | | | | P.M. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *Aug.* 22 | 106 | 141 | 120 | 112 | 129 | 132 | 161 | 165 | 147 | 106 | 167 | 70 | 112 | 152 | 70 | 114 | 53 | 134 | 191 | 82 | 79 | 53 | 100 | 129 |
| 23 | 161 | 132 | 132 | 117 | 112 | 73 | 86 | 82 | 205 | 68 | 100 | 103 | 94 | 117 | 59 | 68 | 126 | 53 | 106 | 82 | 70 | .. | .. | 53 |
| 24 | 55 | 126 | 182 | 117 | 114 | 103 | 134 | 117 | 117 | 117 | 108 | 164 | 94 | 150 | 35 | 141 | 61 | 114 | 123 | 123 | 126 | 103 | 112 | 53 |
| 25 | 20 | 53 | 64 | 44 | 79 | 97 | 120 | 158 | 141 | 106 | 114 | 114 | 112 | 88 | 114 | 106 | 94 | 147 | 97 | 129 | .. | .. | .. | .. |
| 26 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. |
| 27 | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | .. | 150 | 79 | 134 |
| 28 | 94 | 77 | 77 | 129 | 126 | 94 | 82 | 108 | 117 | 129 | 114 | 70 | 82 | 55 | 103 | 103 | 41 | 94 | 103 | 58 | 88 | 24 | 33 | 0 |
| 29 | 11 | 6 | 20 | 24 | 50 | 50 | 35 | 61 | 88 | 53 | 88 | 44 | 62 | 64 | 120 | 47 | 94 | .. | 148 | 58 | 212 | 135 | 115 | 163 |
| 30 | 76 | 87 | 62 | 135 | 110 | 76 | 120 | 130 | 91 | 111 | 130 | 76 | 106 | 101 | 72 | 96 | 115 | 135 | 130 | 197 | 130 | 87 | 104 | 110 |
| 31 | 53 | 29 | 62 | 62 | 91 | 58 | 34 | 34 | 34 | 58 | 38 | 102 | 34 | 87 | 72 | 53 | 125 | 96 | 101 | .. | .. | 85 | 85 | 62 |
| *Sept.* 1 | 165 | 67 | 94 | 97 | 164 | 178 | 162 | 158 | 143 | 164 | 106 | 150 | 166 | 162 | 120 | 97 | 91 | 138 | 141 | 158 | 117 | 62 | 106 | 100 |
| 2 | 120 | 105 | 138 | 158 | 82 | 137 | 143 | 129 | 173 | 109 | 109 | 109 | 85 | 120 | 109 | 105 | 67 | 152 | 73 | 38 | .. | .. | 110 | 97 |
| 3 | 92 | 128 | 101 | 141 | 123 | 119 | 136 | 132 | 136 | 145 | 198 | 0 | 0 | 31 | 57 | 146 | 39 | 57 | 106 | 101 | 18 | 44 | 75 | 61 |
| 4 | 79 | 66 | 79 | 66 | 79 | 128 | 31 | 75 | 66 | 71 | 66 | 53 | 44 | 52 | 66 | 66 | 61 | 57 | 61 | 53 | 141 | .. | 94 | 71 |
| 5 | 59 | 100 | 44 | 38 | 71 | 147 | 123 | 94 | 73 | 165 | 170 | 63 | 41 | 85 | 35 | 100 | 131 | 111 | 94 | 117 | 100 | 155 | 138 | 38 |

rate occurred earlier, at 6 to 7 A.M., and the evening maximum was also earlier, at 7 to 9 P.M. The mid-day minimum, on the other hand, occurred later in the day, between 1 and 2 P.M., and the second minimum was earlier in the day, between 10 and 11 P.M. The absolute values of $O_2$-consumption were distinctly higher than for pugnax. The mean value for the 15-day period was 99.2 ml/kg/hr., and the hourly averages for 4 to 6 crabs ranged from 77 to 117.5 ml/kg/hr. From the minimum daily average value to the maximum involved about a 52% increase.

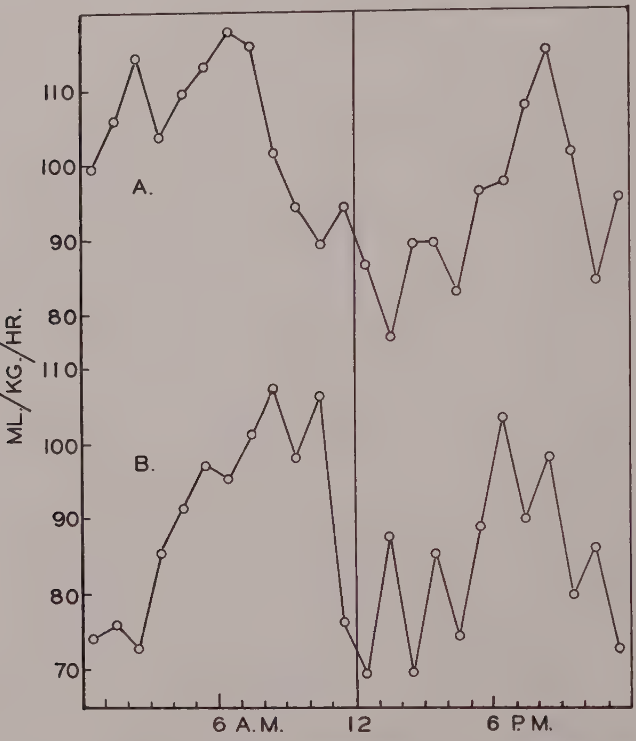

Fig. 4 The diurnal variation in $O_2$-consumption in *Uca pugilator* obtained by randomizing the tidal rhythm for the semilunar period, August 22nd–September 5th, for normal (A) and eyestalkless (B) crabs.

The eyestalkless animals, despite the fact that two consecutive days of data are absent in the 15-day period for them and as a consequence the tidal cycles would tend to obscure the diurnal cycle, also exhibited a clear daily cycle. This is shown in figure 4 B. The primary difference of the cycle from that of the normal crabs was a general displacement of the morning period of increased respiration to a point about three hours later in the day, and an earlier onset of the evening period of increased respiration. It cannot be ascertained at this time to what extent these recorded shifts are due to the incompleteness of data in the 15-day series. The average value for the 13-days of recording was 87.3 ml/kg/hr., with the hourly averages for the 4 to 6 animals ranging from 69.5 to 107.0 ml/kg/hr. From the lowest to the highest value in the average daily cycle, there was a 54% increase.

The results of randomizing the daily cycle of the normal crabs to expose any existing tidal rhythm are plotted in figure 5 A. There is seen unequivocally to be a persistent tidal rhythm of respiration. While the number of hours between the minima in this figure does not suggest a cycle of other than about 12-hour length, the time between the maxima do clearly indicate that the cycle length is probably nearer the expected 12.4 hours. As in U. pugnax, the tidal cycle in this species also seems to have a more or less complex form, with maximum occurring just before the time of a low tide and the minimum near the time of high tide. There also appears to be a more sharply delimited period of very high $O_2$-consumption. From the lowest to the highest hourly value in the average tidal cycle there was a 50% increase. The tidal cycles were therefore just about as effective as the diurnal in producing variations in $O_2$-consumption.

For analysis for a tidal rhythm in the eyestalkless crabs an attempt was made to reduce the specific effect which would be produced by the absence of the two days of data by substituting in their stead the average daily cycle of the 13-day period recorded, but now displaced in the appropriate manner to contribute to the randomizing of the daily cycle. It was realized, however, that these would at the same time tend to diminish the amplitude to any existent tidal rhythm. Since, however, these average daily cycles contributed only about 15% of the data, it was believed that any tidal rhythm which might be present would still be evident by this method. The results of this analysis are plotted in figure 5 B. It must be concluded that these eyestalkless crabs have no persisting tidal rhythm, or if they have any, either the various animals in the population are no longer in phase with one another or

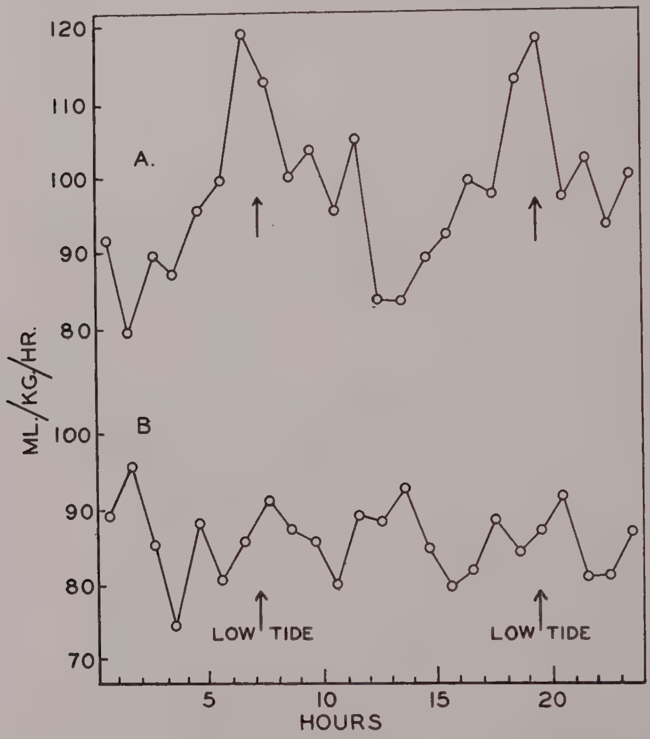

Fig. 5 The tidal rhythm of $O_2$-consumption in Uca pugilator obtained by randomizing the diurnal rhythm for the semilunar period, August 22nd–September 5th, for normal (A) and eyestalkless (B) crabs.

the physiological circuit through which $O_2$-consumption is regulated by the tidally rhythmic mechanism has been destroyed.

## DISCUSSION

The variations in $O_2$-consumption that have been measured in this study of diurnal and tidal rhythms undoubtedly simply reflect the amount of motor activity of the animals. No attempts were made in the course of this work to distinguish any rhythmic variations in such motor activity, per se. It appears unreasonable to postulate that these variations which have been shown to amount to as much as 6-fold to 8-fold differences could have any other origin.

The three 15-day series using *Uca pugnax* revealed average rates of $O_2$-consumption of 39.8, 37.3, and 57.8 ml/kg/hr. over these periods. These three values may reflect significant differences reflecting the rates of metabolism at the experimental 25°C. of animals taken from the field, in late July, early August, and late August and early September, respectively, and hence result from some seasonal acclimatization to temperature. In fact, the order of these (with cooler weather in late August and September) would suggest such a possibility. Another, and possibly more likely explanation is that with the gradual reduction in length of daylight during the course of the three series, there has been a gradual reduction in the duration of the mid-day low rate of $O_2$-consumption, with a resultant higher overall average. This is strongly suggested by the increasing approximation of the morning and evening maxima through the three series. In fact, it is quite possible that an apparent seasonal acclimatization of metabolic rate to temperature might be, at least in part, a result of an alteration in character of the diurnal pattern of activity. However, in view of the fact that before the commencement of the third series the spring scales were reconstructed and increased in their smoothness of action, the respiratory chambers provided with somewhat greater surface area of $CO_2$ absorbent, and the plastic $O_2$-containing sacks altered in form to assure their more uniform collapse, it is not possible to view these differences with as much confidence as would othewise be the case.

Within each of the three series with *Uca pugnax,* however, there appeared to be some systematic variations in total daily $O_2$-consumption of the crabs through the 15-day periods which could not be interpreted readily in terms of simply tidal and diurnal rhythms. The character of these variations was definitely different from one 15-day cycle to another. These differences became evident as systematic ones when one attempted to randomize the data for both the diurnal and tidal by moving the daily data relative to previous days' data at a tidal rate but in a direction opposite that of the tides, in a 29-day analysis. Instead of obtaining a complete abolition of all rhythmicity as had been expected, long-period, low-amplitude variation quite different in character from the tidal and diurnal ones described earlier made their appearance and the general forms were different for each of the three series.

To attempt to describe them further, and to learn possibly whether these were cyclic, and if so the nature of the cycles, the data of series 1 and 2 were combined to provide a continuous 30-day one. This is obviously a lunar period. With these data, the average persistent diurnal and tidal variations were calculated, now without the need of repeating the use of the data to determine the nature of the tidal cycles. The cycles obtained are graphically depicted in figure 6 A and B, respectively. In figure 6 C are illustrated the results of randomizing presumably both the tidal and the diurnal rhythms by moving the daily data of succeeding days with respect to one another at a rate as in the tidal analysis but now in the opposite direction. By this means the daily rhythm scans the 24 hours once and the tidal cycles 4 times. The curves have been smoothed by using as the value for each hour the average of it together with the values for the preceding and following hours in the cycle. The results indicate that the crabs possess an endogenous rhythm of $O_2$-consumption in addition to their tidal (12.4 hour), diurnal (24.0 hour) and semilunar ones. This is responsible for a range of variation in average $O_2$-consumption from 31 to 51 ml/kg/hr., or an increase of 64% from minimum to maximum in the cycle. This third type of rhythm which makes itself apparent in figure 6 C is one with cycles of semilunar or lunar length. There are two general ways in which the results of this figure could have been obtained. One is that there be a lunar rhythm

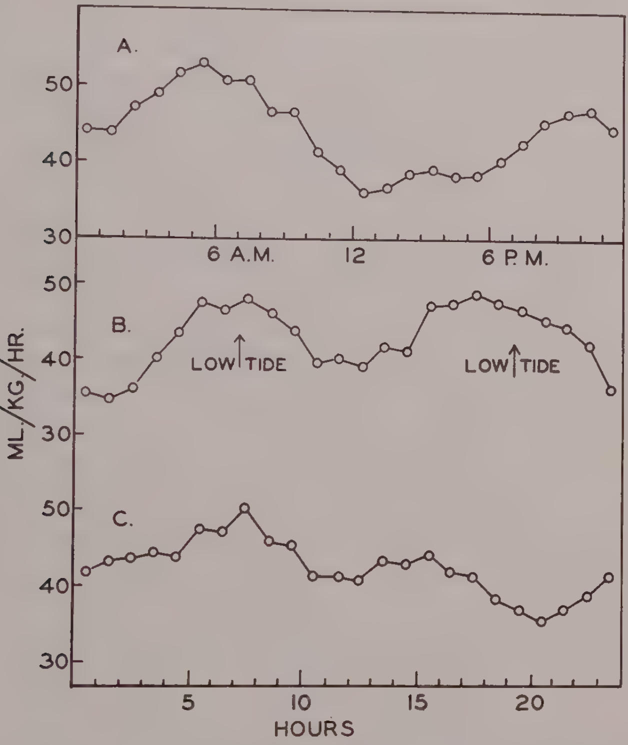

Fig. 6 (A) The smoothed, average diurnal rhythm of $O_2$-consumption for *Uca pugnax* obtained for the two semilunar cycles; (B) the smoothed average tidal cycle for the two series; and (C) a new cycle of variation obtained as a result of randomizing both of the preceding rhythms in a manner described in the text. This demonstrates the existence of a persistent lunar cycle.

in some component of the persistent diurnal rhythm so that as it scans the 24 hours of the columns of data, it traces out the cycle illustrated. The other is that alternate tidal cycles (lunar day or 24.8 hour) exhibit semilunar cycles (14.8 day) of variation in amount of influence on $O_2$-consumption relative to the intervening tidal cycles. Of these two possibilities, the second appears perhaps to be the more plausible in view of the fact that the cycles to be explained could be accounted for in terms of diurnal, tidal, and semilunar cycles already established for the species, except for the added need for the integration within the organism of successive pairs of tidal cycles. The first possibility, on the other hand, although it cannot be excluded, would appear to call for a more direct lunar influence than the second one. Even in the event that these results were to be explained in terms of the possession of a rhythm of lunar-day cycles, the crabs would still by periodic reinforcement of the diurnal by the lunar-day cycles have a persistent lunar rhythm. Therefore, it appears quite definite that figure 6 C indicates the presence of a persistent 30-day or lunar rhythm in these crabs.

The results clearly indicate that the rate of $O_2$-consumption of *Uca pugilator* is about 70% greater on the average than for *U. pugnax.* It is interesting to attempt to correlate this with their normal habits. The burrows of *U. pugilator* are usually much nearer the high-tide level at Chapoquoit where the animals were collected than are the burrows of *U. pugnax,* and consequently they have a much greater distance to travel to the normal feeding grounds near the low tide level. In the field, *U. pugnax* is observed in general to feed closer to its burrows, and are seldom found swarming at the water front at low tide as is pugilator.

It is quite obvious from these results that it is essential that one has the average rate of $O_2$-consumption for a 15-, or even possibly a 30-day period before one has included the complete range of variation that is to be found in the average animal in the population due to predictable endogenous regulating factors. But using such a 15-day period, it is readily apparent that removal of the eyestalks with their included X-organs and sinus glands has resulted in an average reduction of about 12% in the rate of $O_2$-consumption. These measurements were made upon crabs whose stalks had been removed 1 to 6 days previously.

Another significant conclusion that has come from this work is the fact that the diurnal rhythm of $O_2$-consumption can persist in the absence of the eyestalks. Therefore, an hypothesis which was once advanced, namely that the endogenous diurnally rhythmic mechanism was located in the sinus gland is untenable. Furthermore, it is now clear that nothing else of the eyestalk, glandular or nervous is essential to the basic rhythmic mechanism of diurnal rhythmicity. Although the phases of the diurnal rhythm are very readily reset by light stimuli, presumably through the eyes, the vigorous non-differential stimulus of section of the optic nerves in the course of eyestalk removal has clearly not altered the phases of the diurnal rhythm. The pattern of the cycles has been changed. The times of the maxima and minima have been shifted slightly relative to one another. These latter differences may be due to the removal of the eyestalk endocrine glands which may normally participate in the regulation of these particular rhythmic overt responses. In fact, the absence of a tidal rhythm of $O_2$-consumption in these stalkless forms may be directly a consequence of the tidal rhythm being normally the result of an induced rhythmic secretion of endocrines from the X-organ or sinus gland.

It is seen from the results obtained in this work that the fiddler crab has intrinsic rhythms of a number of frequencies. These include ones with 12.4-hour and 24.0-hour cycles. It has been shown in addition that accurate cycles of 14.8 days and either 24.8 hours or 29.5 days are also present. It seems quite clear that the 14.8-day cycles are the results of periodic reinforcement of the 24.0-hour cycles by the 12.4-hour ones. The 30-day cycle which seems clearly to be present may result from the simultaneous presence of 24.8-hour and 24.0-hour cycles or may be a basic one like the tidal and the diurnal ones.

It seems extremely probable that all of the rhythms reported in this paper have their frequencies independent of temperature over at least a moderate temperature range. This seems so in view of the fact that all of these cycle-lengths corresponded so closely with the lengths of the physical cycles despite the fact that all the values were obtained at an arbitrarily selected temperature (25°C.) and under otherwise constant conditions. A temperature of 25°C. was undoubtedly somewhat higher than the average body temperature of the crabs during the summer in their natural habitat.

## SUMMARY

1. The rate of $O_2$-consumption of the fiddler crab, *Uca pugnax,* has been determined continuously over one lunar period, and over another period of semilunar length.

2. An analysis of the records obtained reveal that this crab has endogenous rhythms of tidal (12.4 hours), diurnal (24.0 hours), semilunar (14.8 days), lunar (29.5 days) cycles and possibly even lunar-day (24.8 hours) ones.

3. The diurnal component leads to a maximum of $O_2$-consumption at 6–8 A.M., and a second lesser maximum about 10–11 P.M. A minimum occurs about noon, and a lesser one about midnight.

4. The tidal component provides a minimum rate about the time of high tide in the habitat where the crabs were collected, and a maximum shortly before the time of low tide.

5. A 14.8-day cycle is apparent as the result of periodic reinforcement of one another by the two preceding cycles.

6. Either a lunar or a lunar-day cycle is also present. The observed lunar cycle can be explained in terms either of periodic reinforcement of one another by the diurnal and a lunar-day cycle, or the crabs actually possess an independent endogenous cycle of lunar length.

7. Comparable endogenous daily and tidal rhythms of $O_2$-consumption were also found in *Uca pugilator.* The cycles of the rhythms in this species differed slightly but significantly in form from those of *Uca pugnax.*

8. Eyestalkless *Uca pugilator* exhibited a persistent daily rhythm of $O_2$-consumption with an amplitude as great as that of normal crabs. The daily pattern differed slightly in form from that of normal animals.

9. Eyestalkless *Uca pugilator* lacked a persistent tidal rhythm of $O_2$-consumption.

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# TRANSPHOSPHORYLATION IN ECHINODERMS[1]

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The phylum Echinodermata contains forms that have been shown by ordinary analytical methods to contain both of the phosphagens, arginine phosphate (AP)[2] and creatine phosphate (CP) occurring either singly or together. Members of the classes Crinoidea [*Antedon bifida* (Baldwin and Yudkin, '50), *A. mediterranea* (Baldwin, '37)], *Asteroidea* [*Marthasterias glacialis* (Needham, et al., '32)], and Holothuroidea [*Holothuria tubulosa, Stichopus* sp. (Meyerhof, '28), *Synapta inhaexens* (Needham, et al., '32), *Thyone briareus* (Baldwin and Yudkin, '50)] which have been examined have been shown to contain only AP with one report questionable. Of the Echinoidea both AP and CP have been found together in the jaw muscles of *Echinus esculentus* (Baldwin and Yudkin, '50) and *Paracentrotus lividus* (Needham et al., '32), and AP alone in *Arbacia punctulata* (Baldwin and Yudkin, '50). Of the Ophiuroidea [*Ophiothrix fragilis, Ophioderma brevispina* (Baldwin and Yudkin, '50), *O. longicauda* (Baldwin, '37)] examined, all assayed for CP alone. In all these cases the characteristic hydrolysis rate in acid in the presence of molybdate was used as a differentiating assay. Meyerhof ('28) in addition, estimated arginine with arginase.

The final step in the synthesis of the phosphagens is the phosphorylation of the appropriate guanidine base by adenosine triphosphate (ATP) (Lohmann, '35). This equilibrium is catalyzed by the enzymes creatinephosphokinase and argininephosphokinase, specific for each base (Lehmann, '35; Ochoa, '38). Thus, the occurrence of these enzymes in muscle is regarded here as *a priori* evidence for the occurrence of the individual phosphagen. Moreover, the specificity of the enzyme serves to determine the specific guanidine base involved when assay by hydrolysis can leave doubt (Baldwin and Yudkin, '50). Extracts from vertebrate muscle have been found to phosphorylate creatine but not arginine (Lehmann, '35; Ochoa, '38), and muscle extracts from crab (Lehmann, '35), lobster (Ochoa, '38), scallop (Yudkin, '50), and sea cucumbers (Baldwin and Needham, '37) could phosphorylate arginine but not creatine. Both phosphagens have been found in the sea urchins *P. lividus* and *Sphaerechinus granularis,* and muscle extracts of these forms phosphorylated both bases (Baldwin and Needham, '37).[3]

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[1] Contribution number 220 from Bermuda Biological Station for Research, Inc. This work was supported by grants from the Graduate School of Northwestern University and the Bermuda Biological Station.

[2] The following abbreviations are used: AP, arginine phosphate; CP, creatine phosphate; ATP, adenosine triphosphate; $P_7$, pyrophosphate P.

In this paper the study of the phosphokinases has been extended to 4 classes of the echinoderms in order to identify the specific phosphagens present. The asteroids and holothuroids phosphorylate only arginine, the echinoids phosphorylate both arginine and creatine or only arginine, and the ophiuroids phosphorylate only creatine.

## EXPERIMENTAL

*Asterias forbesi* was shipped from Woods Hole to Evanston in January and used upon arrival. *Arbacia punctulata* was collected in Woods Hole and used there within a day. All the other animals were collected in Bermuda and kept in aquaria for not more than three days before being used. With the exceptions noted above, these experiments were carried out at the Bermuda Biological Station for Research. Echinoid nomenclature is that of Mortensen ('28–'51); ophiuroids and holothuroids were named in accordance with the descriptions given by Clark ('33). I am grateful to Dr.

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[3] Preliminary experiments have indicated that in such a form (*Lytechinus*) there exist two separate phosphokinases rather than a single enzyme transphosphorylating to either arginine or creatine.

Elisabeth Deichmann for her expert aid in identifying the ophiuroids.

Extracts were made by triturating the appropriate tissue with 3 to 5 times its weight of cold 0.9% KCl solution and about 3 gm of reagent sea sand. This brei was lightly centrifuged to remove the sand and debris, and the supernatant was dialyzed in the cold for 36 hours against three 3 1. portions of 0.4% KCl solution. The extract was used immediately after light centrifuging. Its appearance depended on the animal and the tissue used.

To test phosphokinase activity, the following additions were made to a 15 ml centrifuge tube in the order given: 0.2 ml $Na_2ATP \cdot 3H_20$ (Pabst) containing 1 mg $P_7$/ml; 0.5 ml barbital buffer, 0.1 M, pH 7.8; 0.1 ml 0.5 M NaF; 0.2 ml 0.09 M creatine hydrate; 0.2 ml 0.09 M L-arginine hydrochloride brought to pH 8 with NaOH; 0.8 ml tissue extract. Omissions were made by substituting an equal volume of water. Controls were run with ATP omitted and with creatine and arginine omitted. At first, 0.02 ml of 0.5 M $MgCl_2$ was also added, but the only apparent effect was to inhibit transphosphorylation in this crude system, so in all experiments reported here $Mg^{++}$ was omitted. The mixtures were incubated for 90 min. at 27°C.

Phosphate analyses were made by the method of Fiske and SubbaRow ('29) adapted to a Klett-Summerson colorimeter with a red (no. 66) filter. At the end of incubation the tubes were cooled to 3°C. and 2 ml of cold 8% trichloroacetic acid was added. The precipitated protein was spun down and discarded and the supernatant was brought to pH 8.2 with NaOH and phenolphthalein as indicater. Fractionation of the acid-soluble phosphates was done with barium in the usual manner (Umbreit, Burris, and Stauffer, '49). In a few cases, however, 50 γ P as orthophosphate was added to aid in the precipitation of the organic pyrophosphates.

The barium-soluble fraction was analyzed for the phosphagens after clearing it of $Ba^{++}$ with $Na_2SO_4$. Half of this solution was used for estimating CP by the method of Fiske and SubbaRow ('29), and the other half was used for estimating total phosphagen. Assay of total phosphagen was accomplished by adding 11% of the sample volume of 20% trichloroacetic acid and heating the mixture on a boiling water bath for 1 min. Under these conditions both CP and AP are hydrolyzed completely, and phosphate analysis indicated the total phosphagen present (Lohmann, '36). The difference between the total phosphagen and the CP determination is the amount of AP present. Previous determinations of the rate of hydrolysis of AP indicate that under the conditions (0.5 N $H_2SO_4$ and 0.25% ammonium molybdate for 20 min. at room temperature) required to hydrolyze all the CP, 10% of the AP is hydrolyzed also (Baldwin and Yudkin, '50). Therefore the analysis of CP will be high and the analysis of AP will be low. The barium-insoluble fraction was analyzed for inorganic phosphate and for pyrophosphate ($P_7$).

TABLE 1

*Transphosphorylation in Echinometra lacunter*

Extract made from 2 gm interpyramidal muscle from jaws of 7 specimens ground with 10 ml 0.9% KCl. Treated and incubated as described in text. All tubes contain in 2 cm³ 1.7 mM ATP, 25 mM NaF, 25 mM buffer and tissue extract equivalent to 0.18 gm tissue. Fifty gamma P as inorganic phosphate added before Ba precipitation. Creatine and arginine concentrations were 9 mM. Incubated 90 min. at 27°C.

| EXPERIMENT | ADDITIONS | $\gamma$ P PER TUBE AS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Phosphagen | CP | AP[1] | Inorganic P | $P_7$ | Total P[2] |
| 1 | None | 5 | 5 | 0 | 61 | 154 | 220 |
| 2 | Creatine | 26 | 26 | 0 | 54 | 129 | 209 |
| 3 | Arginine | 65 | 10 | 55 | 50 | 76 | 191 |
| 4 | Creatine and arginine | 64 | 17 | 47 | 50 | 85 | 199 |

[1] AP = Phosphagen — CP.

[2] Total P = Phosphagen + inorganic P + $P_7$.

## RESULTS

Table 1 shows the results of an experiment with the echinoid, *Echinometra lacunter*. Atypical in this experiment is the pronounced inhibition of apyrase activity. In most cases the prolonged dialysis and NaF inhibit the hydrolysis of ATP only about 60%. For reasons mentioned above, the value of 10 γ P as CP in table 1, experiment 4 is likely to be closer to 5 γ P and consequently the value for AP should be increased to 60 γ P. The formation of 5 γ P as CP in the absence of added creatine in table 1, experiments 1 and 3, can perhaps be attributed to creatine present which was not dialyzed away. An additional source of error in the phosphagen determinations is the presence of finely divided $BaSO_4$ carried over from clearing the barium-soluble fraction of $Ba^{++}$. It is evident from table 1 that about 25% of the phosphate added as $P_7$ is lost either to the precipitated protein or to phosphate fractions not determined. Complete recovery would be 250 γ P, of which at least 50 γ is inorganic phosphate added during the course of the analysis to aid in the precipitation of the adenyl pyrophosphates.

The results obtained with the animals employed in this study are summarized in table 2. The analytical value for CP is corrected by substracting from it both the value for CP formed in the absence of added creatine and also 10% of the value for AP, where it was formed. The value for AP in table 2 was obtained by subtracting the AP formed in the absence of arginine from the analytical value for AP and then increasing this difference by 10%. These corrections amount to less than 10 γ P and so the error in the data in table 2 is of the order of ± 1 γ P.

## DISCUSSION

The enzyme assays shown in table 2 confirm the results of earlier workers who have shown that the asteroids and holothuroids contain AP, the echinoids contain both AP and CP, and the ophiuroids contain CP alone. Upon finding both phosphagens in *Paracentrotus* and in *Balanoglossus*, (Needham, et al., '32) indicated that they had confirmed with biochemical evidence the origin of chordates from echinoderm stock proposed by Bateson (1884) through larval resemblances. Complete discussions of this aspect are available (Baldwin, '33; Needham and Needham, '31–'32).

TABLE 2

*Arginine phosphate and creatine phosphate formed by tissue extracts*

Values corrected as described in text. All tubes contain in 2 cm³ 1.7 mM ATP, 25 mM NaF, 25 mM barbital buffer pH 7.8, and tissue extract. Creatine and arginine concentrations were 9 mM. Incubated 90 min. at 27°C.

| ANIMAL | TISSUE | TISSUE WEIGHT | ADDITIONS | γ P PER TUBE AS | |
|---|---|---|---|---|---|
| | | | | AP | CP |
| | | *gm* | | | |
| Asteroidea | | | | | |
| *Asterias forbesi* | Muscles of arms | 0.40 | Creatine | 0 | 1 |
| | | | Arginine | 0 | 26 |
| | | | Creatine and arginine | 1 | 30 |
| Holothuroidea | | | | | |
| *Stichopus moebii* | Longitudinal muscles | 0.27 | Creatine | 0 | 1 |
| | | | Arginine | 0 | 74 |
| Echinoidea | | | | | |
| *Diadema setosum* | Interpyramidal muscles of jaw | 0.32 | Creatine | 0 | 0 |
| | | | Arginine | 0 | 15 |
| *Lytechinus variegatus* | | 0.16 | Creatine | 32 | 0 |
| | | | Arginine | 3 | 17 |
| *Tripneustes esculentus* | | 0.16 | Creatine | 7 | 0 |
| | | | Arginine | 1 | 11 |
| *Echinometra lacunter* | | 0.16 | Creatine | 21 | 0 |
| | | | Arginine | 0 | 60 |
| | | | Creatine and arginine | 7 | 52 |
| *Arbacia punctulata* | | 0.06 | Creatine | 0 | 1 |
| | | | Arginine | 1 | 90 |
| | | | Creatine and arginine | 0 | 86 |
| Ophiuroidea | | | | | |
| *Ophionereis reticulata*[1] | Whole arms | 0.27 | Creatine | 24 | 0 |
| | | | Arginine | 0 | 0 |
| *Ophiocoma echinata* | | 0.55 | Creatine | 8 | 0 |
| | | | Arginine | 0 | 0 |
| *Ophiomyxa flaccida* | | 0.32 | Creatine | 12 | 0 |
| | | | Arginine | 3 | 0 |

[1] Analysis (Baldwin and Yudkin, '50) of whole *O. reticulata* showed it to contain 4.45 mg of P as CP per 100 gm tissue and no AP.

If it is true that the hemichordates, as represented by *Balanoglossus*, contain both phosphagens, as do the echinoids, the relationship between them can be established. Recent assays, however, have demonstrated the existence of only CP alone in two species of *Saccoglossus*. Moreover, the work of Needham, et al. ('32) whose results indicated the presence of both CP and AP in *Balanoglossus* has not been confirmed. According to a personal communication from E. Baldwin and K. R. Rees, some preliminary experiments carried out in the summer of 1951, using specimens of *Balanoglossus* obtained from the same vicinity as those collected by Needham, et al. ('32), evidence for the presence of CP was again obtained, but this time with no indication that AP is present. The conclusions reached by Needham, et al. ('32) some 20 years ago are thus open to serious doubt. Because the ophiuroids definitely contain only CP, there is more reason to postulate that the echinoids are intermediate between the classes of its own phylum rather than between phyla. The occurrence of CP alone in the hemichords would not indicate any special relationship between the hemichords and the echinoids.

The distribution of phosphagens within the phylum Echinodermata suggests that the ophiuroids are more closely related to the echinoids than to the other 4 extant classes. For lack of positive data the class Crinoidea is not considered in this discussion. Larval stages (pluteus) of the echinoids and ophiuroids are similar to each other as are the larval stages (auricularia) of the asteroids and holothuroids. The two classes arising from the auricularian larva contain AP only; those coming from the pluteus larva contain CP in some forms. This relationship is supported by Bergmann ('49) in his study of the predominant patterns of the occurrence of certain sterols in these forms. On the other hand, Fell ('48) deplores the use of recapitulation theory in tracing the phylogeny of echinoderms. He has emphasized that the larval characteristics of closely related forms in the same class are often quite different and, in fact, one can obtain larvae of echinoderms in two different classes that look more alike than two of forms in the same class. For this reason he feels that it is unwise to relate echinoderms to each other by using only larval resemblances and that it is even more extravagant to relate the hemichords to the echinoderms in this manner. Fell also demonstrates that relationships concluded on larval resemblances do not at all agree with the paleontological record. These arguments notwithstanding, if the larval forms are indicative of the phylogeny of the individual classes, it can be supported on a biochemical basis. At this time no support on the same basis can be given concerning the origin of chordates.

A further division of the Echinoidea themselves may be made on the basis of the distribution of the phosphagens in the few species at hand. Those forms so far investigated which have been shown, here and in the other works cited, to contain both AP and CP have been placed by Mortensen ('28–'51) in the order Camarodonta. *Arbacia* and *Diadema,* however, both of which contain only AP are in the orders Stirodonta and Aulodonta, respectively. It will require extension of this work to a greater variety of genera than presented here fully to substantiate this suggestion. But on the basis of the evidence at hand, one might predict that other arbaciids would contain only AP. When the orders have been more fully explored, a pattern may emerge.

Present evidence can be interpreted to indicate that the possession of AP by an animal is a more primitive characteristic than a similar situation involving CP. In this connection it may be noted that Bregman and Yudkin (unpublished) have found that muscle from *Limulus polyphemus* contains AP and transphosphorylates to arginine alone. Recently Wald ('52) has restated Meyerhof's ('41) view that the commutation from arginine to creatine is the result of a single mutation. At least three enzymes, however, are implicated in the synthesis of creatine from arginine and a 4th would be necessary to obtain CP from creatine. Consequently, the change from AP to CP may be more complicated than one which is under the control of a single gene presumably controlling a single enzyme. Van Thoai and Robin ('51) have demonstrated that certain invertebrates, known to use only AP, lack enzymes necessary to methylate glycocyamine with methionine or betaine but the enteropneust, two echinoids, and the ophiuroid examined can synthesize creatine from these precursors. An interesting exception is the scallop, *Pecten maximus,* which can synthesize creatine but can transphosphorylate only to arginine (Yudkin, '50). By chromatographing muscle extracts Van Thoai and Robin ('51) found glycocyamine in the sea urchin (*Paracentrotus*) but none in the scallop and the spiny lobster (*Palinurus vulgaris*). Thus it appears that the scallop can methylate glycocyamine but cannot synthesize it from arginine since neither glycocyamine nor creatine is present in the muscle of this form.[4] Cantoni ('51, '52) has shown that transmethylation from methionine to glycocyamine is performed in two steps mediated by two enzymes. It is possible that all invertebrates have the enzyme required to produce "active" methionine from methionine and ATP, but that in those which do not synthesize creatine from glycocyamine, the second, transmethylating enzyme may be absent. To substitute CP for AP an animal must acquire two or more of three enzymes, a transamidinating system (Borsook and Dubnoff, '41), a transmethylating enzyme (Cantoni, '51, '52), and a transphosphorylating enzyme (Lohmann, '34).

Wald ('52) has suggested that in the echinoderms the change from CP back to AP may have occurred. This would involve the loss of one enzyme in the chain leading to the synthesis of creatine from arginine and the exchange of creatinephosphokinase for argininephosphokinase. On this basis the apparent ability of *Pecten* to synthesize creatine from glycocyamine (Van Thoai and Robin, '51) might be associated with a previous occurrence of creatine in the molluscs. In any event, this change would mean the loss of two enzymes and the gain of one. The exchange of CP for AP and *vice versa* does not seem, therefore, to be a simple, one-step, substitution but rather a complex series of related events must take place for it to occur.

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[4] This, however, does not exclude the possibility that creatine can be synthesized in the testes of some invertebrates which have only AP in their muscle (Greenwald, '46).

Generally, it is recognized that the arbaciids and diadematids are more primitive in the class Echinoidea than are the members of the order Camarodonta (Mortensen, '21). Thus the situation that *Diadema* and *Arbacia* both have only argininephosphokinase whereas the camarodonts have both argininephosphokinase and creatinephosphokinase may be a reflection of the possession of only AP as the most primitive condition.

## SUMMARY

1. The occurrence of creatinephosphokinase and argininephosphokinase in extracts from several echinoderms was determined.

2. The single asteroid and holothurian examined contained only argininephosphokinase.

3. Of the 5 echinoids examined, two contained argininephosphokinase and three contained both phosphokinases.

4. The three ophiuroids examined contained only creatinephosphokinase.

5. The significance of the distribution of the phosphagens is disscussed with reference to echinoderm phylogeny.

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# THE BLOOD-BRAIN BARRIER — THE EFFECT OF ACIDIC DISSOCIATION CONSTANT ON THE PERMEATION OF CERTAIN SULFONAMIDES INTO THE BRAIN[1]

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Previous studies on the blood-brain barrier which have utilized cocaine as a tracer agent have indicated that the permeability of this barrier was increased in such conditions as cerebral concussion and electric shock therapy (Aird and Strait, '44; Aird et al., in preparation; Aird et al., '52; Strait et al., '41). Since observations based upon a single tracer agent, however, do not permit general conclusions with respect to the permeability of the blood-brain barrier, it became desirable to compare these initial results obtained with cocaine, with results obtained with other tracer agents. This, in addition to the laboriousness of the cocaine analysis, led us to initiate studies of cerebrovascular permeability with other compounds.

A review of the literature strongly suggested a similar inadequacy in the techniques used in the majority of the previous studies that have been made on the blood-brain barrier. It would appear that studies of the permeability of this important neurophysiological mechanism, based upon single tracers selected at random, might contribute little to our understanding of the barrier function as a whole. The ques-

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tion arises as to what factors regulate the permeability of this barrier, and how an adequate determination of barrier permeability may be made. Such considerations have indicated to us the desirability of a more basic approach.

A systematic study of barrier function with the use of a series of related compounds would appear to be essential if such factors as the acidic dissociation constant, molecular weight and aqueous and lipid solubility are to be evaluated as determinants of barrier permeability. The present report is concerned with the initial results obtained with the sulfonamide group of compounds. In addition to possessing the wide range of physical characteristics required for the study, this group has the advantage of (1) being relatively non-toxic and safe for experimental study and possibly lending itself to clinical use should this prove desirable, (2) penetrating the barrier in variable degrees, and (3) lending itself to quantitative measurement by a simple, accurate and direct method.

Fisher, Troast, Waterhouse and Shannon ('43) studied the relation of the acidic dissociation constant (pKa) to sulfonamide permeation into brain as part of a comprehensive survey of the physiological distribution of the sulfonamides. These authors concluded that the pKa values of the sulfonamide compounds bore an important relationship to the permeability of these compounds through the blood-brain barrier. Since they felt, however, that the relationship was not too precise and probably but one aspect of molecular structure, we considered further investigation of this question to be desirable. Doctor Shannon, indeed, expressed to one of us (R. B. A.) the opinion that further work along this line, utilizing the sulfonamide group of compounds, would be worthwhile. It should also be mentioned that the present study differs from the work of Fisher et al. in certain aspects. Their carefully controlled procedure allowed the measurement of distribution under equilibrium conditions whereas our experiments were terminated before maximum brain uptake had occurred. Since certain of the sulfonamides pass through the barrier with considerable ease, it was hoped that experiments involving measurements of the rate of passage would be more sensitive than final distribution levels in determining the relationship between the pKa values of the sulfonamides and their ability to permeate the barrier. It was felt that this procedure might also reduce the effect of the binding of the sulfonamides or their localization within the cells. Another point of difference arises in that the procedure of Fisher et al. required light nembutal anesthesia shortly before administration of the test sulfonamide, whereas our methods involved nembutal anesthesia in the terminal 5 minutes only. That this may be a point of considerable importance is suggested by the study of Teague and Perdue ('48) who noted an increase in the three-hour cerebrospinal fluid to blood ratio for sulfathiazole when dogs were maintained under dial-urethane or ether anesthesia.

## METHODS

Male rats of the Long-Evans strain, weighing at least 200 and usually about 300 gm, were used throughout the study. Mature animals were selected in order to avoid the variability sometimes noted in younger animals. The rats were maintained on a standard diet, and care was taken to minimize variations of an environmental nature.

A group of sulfonamides that had a wide range of pKa values were chosen as test compounds.[3] At the start of the experimental procedure each rat was given an intraperitoneal injection of approximately 6.0 ml of 0.8% solution or suspension of the sulfonamide to be tested. Exact dosage was not found to be critical in the case of three of the sulfonamides used [$N^1$-(β hydroxyethyl)sulfanilamide, sulfapyridine, and sulfanilamide], since a many-fold variation in dosage resulted in no difference in the effect on the brain to blood ratio values. Fifty-five minutes after the sulfonamide injection, the animal

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[3] The authors wish to thank the Calco Chemical Division of the American Cyanamid Company for their generosity in supplying us with most of the sulfonamides used in this study.

was anesthetized by an intraperitoneal injection of nembutal (5–10 mg per 100 gm of body weight). Five minutes later, 5–6 ml of blood were drawn from the inferior vena cava with a syringe wet with heparin. The animal was sacrificed immediately thereafter by exsanguination, in order to obtain the sulfonamide content of the brain at a level related in time to the blood sample level, and to rid the brain of as much blood as possible. The brain was then removed, rapidly rinsed with distilled water, blotted, weighed and homogenized in a Potter-Elvehjem glass homogenizer in a known volume of water.

One milliliter samples of blood and plasma were deproteinized at dilutions of either 1:25 or 1:50 by volume according to the method of Bratton and Marshall ('42). Brain aliquots were deproteinized at dilutions ranging from 1:20 to 1:100 by weight; the final concentration of trichloroacetic acid being 6%. Total sulfonamide was determined by the method of Bratton and Marshall ('42). Optical density measurements were made using a Beckman model DU spectrophotometer set at a wave length of 545 mμ.

## RESULTS AND DISCUSSION

The data for the various sulfonamides are presented as ratios of brain to blood concentrations. Control studies showed that the conjugation of sulfonamide which occurs in one hour had no significant bearing on the results. In relating the concentrations of the sulfonamides in brain and blood at disequilibrium it is essential to consider such factors as the rate of absorption, excretion and distribution of the sulfonamides insofar as they affect the blood concentration-time curve. The relative uptake of the sulfonamides in the brain is conditioned in part by the shape of the uptake curve for blood. This influence diminishes as equilibrium is approached. The comparison of the brain-blood ratios for different sulfonamides at conditions of disequilibrium as made in this study is of value only for the following reasons: Absorption from the intraperitoneal cavity is rapid, and the peak blood concentration is usually reached within the first half hour after administration of the sulfonamide. The blood concentration-time curves of the various sulfonamides thus are essentially similar. Furthermore, by the time the animal is sacrificed the concentration of the sulfonamides in the blood is relatively stable, that is, the rate of change of the blood concentration is fairly slow. Both factors, namely, the similarity of the blood concentration-time curves and the reduced rate of change of blood concentration at the time of sacrifice, combine to produce a variable whose magnitude is small compared to the difference between ratios. This picture is also reasonably true for those sulfonamides injected in part as a suspension, since the blood uptake apparently reflects in the main that portion of the dose which is in a soluble form. As implied previously, the ratio has no relation to the magnitude of the blood concentrations achieved.

The brain to blood ratios obtained are listed in table 1. Each value is the mean of the results of at least 4 determinations.[4] It will be noted that those sulfonamides whose pKa values [5] are below 8 have a brain to blood ratio of approximately 0.1. Residual blood in the brain probably accounts for some part of these low values. The ratios increase through the range of pKa from 8 to 10.5, then decrease as the pKa value further increases. It thus appears that those sulfonamides which are largely negative at the pH of blood pass into the brain with great difficulty (cf. Friedmann, '42) whereas those that are slightly negative (0.01 to 10% ionized) pass into the brain fairly easily. As the pKa value increases further, the rate of penetration falls.

The plotted data of our rate experiments presents a pattern, which is somewhat similar to that obtained in the equilibrium studies of Fisher et al. ('43). Our data, however, show a somewhat closer correlation between barrier permeability and the pKa values of the agents studied than was the case in the studies of Fisher et al. ('43). The latter authors concluded that molecular configuration, apart from its acidic properties, was an important factor influencing the permeability of the blood-brain barrier. Their conclusions were drawn in part from a consideration of the distribution of the sulfonamides in the cerebrospinal fluid and from the low ratio values of certain sulfonamides with high pKa values, such as $N^1$-ethanol-sulfanilamide and sulfaguanidine. While our results (limited to data on the blood-brain barrier and involving no data on the blood-cerebrospinal fluid barrier) do not contradict this conclusion, the similarity of a curve of our data (pKa values plotted against brain to blood ratios) to an

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[4] The data relating the brain to plasma ratio to sulfonamide pKa values, which are not included, show a similar correlation.

[5] The pKa value of Compound 3 was taken from Lange's Handbook of Chemistry, sixth edition; the values of Compounds 14 and 16 were furnished us by the American Cyanamid Company; and the remainder were taken from the paper by Bell and Robin ('42).

TABLE 1

*The relation of pKa to one hour brain to blood ratio*

| | SULFONAMIDE | pKa | $\frac{\text{brain–mg/100 gm}}{\text{blood–mg/100 ml}}$ |
|---|---|---|---|
| 1. | $N^1$-sulfanilyl-sulfanilamide | 2.89 | 0.13 |
| 2. | Sulfanilyl-cyanamide | 2.92 | 0.14 |
| 3. | Sulfanilic acid | 3.19 | 0.11 |
| 4. | $N^1$-sulfanilyl-glycine | 3.52 | 0.12 |
| 5. | $N^1$-benzoyl-sulfanilamide | 4.57 | 0.06 |
| 6. | $N^1$-acetyl-sulfanilamide | 5.38 | 0.12 |
| 7. | 2-sulfanilamido-pyrazine | 6.04 | 0.09 |
| 8. | Sulfadiazine | 6.48 | 0.12 |
| 9. | Sulfamerazine | 7.06 | 0.14 |
| 10. | Sulfathiazole | 7.12 | 0.11 |
| 11. | Sulfamethazine | 7.37 | 0.11 |
| 12. | $N^4$-sulfanilyl-sulfanilamide | 7.85 | 0.11 |
| 13. | Sulfapyridine | 8.43 | 0.36 |
| 14. | $N^1$-(1-naphthyl)sulfanilamide | 9.6 | 0.60 |
| 15. | $N^1$-o-tolyl-sulfanilamide | 9.96 | 0.72 |
| 16. | $N^1$-octadecyl-sulfanilamide | 10.4 | 1.0[1] |
| 17. | Sulfanilamide | 10.43 | 0.62 |
| 18. | $N^1$-($\beta$ hydroxyethyl)sulfanilamide | 10.92 | 0.25 |
| 19. | Sulfaguanidine | weak | 0.07 |
| 20. | $N^1$-sulfanilyl-$N^3$-aminoguanidine | weak | 0.08 |
| 21. | $N^1$-($\beta$ hydroxyethyl)$N^1$-methyl-sulfanilamide | base | 0.45 |
| 22. | $N^1$,$N^1$-di($\beta$ hydroxyethyl)sulfanilamide | base | 0.15 |

[1] Exact value subject to some error because of low concentrations achieved.

enzyme pH-activity curve suggests one possibility by which pKa might have considerable bearing upon the rate of sulfonamide penetration into the brain *throughout* the pKa range covered. The ability of those sulfonamides which have low pKa values to dissociate a hydrogen ion at a physiological pH would appear to be the predominant factor in determining their lack of entry into brain.

Some authors (Hawkins and Lawrence, '51; Davis, '43) have suggested that the differences in the concentration of sulfonamides found between plasma and the brain or cerebrospinal fluid can be accounted for by the binding of sulfonamide by plasma proteins. This is supported by the fact that observed differences between plasma and brain concentrations under conditions of equilibrium have often approximated the amount found to be bound by plasma proteins. Kummig and Weselmann ('42) concluded that the sulfonamide-albumen combination is an absorption phenomenon which breaks down at cell boundaries. A comparison of our unpublished brain to plasma ratios with the data on plasma binding that is available in the literature indicates that plasma binding may possibly have a limiting effect on the ratio values for sulfonamides in the pKa range from 7 to 10.5, but is not a factor influencing the ratio values of sulfonamides with pKa values above or below this range.

If the molecular weights of the sulfonamides are plotted against the brain to blood ratios, a random distribution is observed. A similar plotting of lipoid solubility against the brain to blood ratio shows no correlation. The latter observation agrees with the results of many workers in the sulfonamide field who have found no relation between sulfonamide distribution and the water or lipoid content of various tissues.

## SUMMARY

A correlation has been noted between the acidic dissociation constants of a number of sulfonamides and their rates of permeation into the brain of mature, male, Long-Evans rats.

Those sulfonamides which are largely negative at the pH of blood pass through the blood-brain barrier with difficulty. Those sulfonamides which are slightly negative penetrate the barrier fairly easily, while those sulfonamides which are essentially neutral gain entrance into the central nervous system at a slower rate. Possible explanations for the observed behavior are considered in the text. No correlation was found between either the molecular weight or lipoid solubility of the sulfonamides and their distribution as indicated by their ratios of brain to blood concentrations.

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# COMMENTS
# AND
# COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

## ELECTRICAL RESPONSES OF INSECT DORSAL OCELLI[1]

PHILIP RUCK
*Department of Zoology, University of California, Los Angeles*[2]

The recent demonstration (Wellington, '53) that dorsal ocelli are by themselves capable of directing a phototaxis in *Sarcophaga aldrichi* indicates that the functional role of these sense organs is not solely that of augmenting the general excitatory state, i.e., that of stimulatory organs (Wolsky, '33). Neither are the electrical responses of dorsal ocelli confined to the type Parry ('47) described for *Locusta migratoria* in the only published electrical study of these receptors. Parry found that on illumination of an ocellus an electrode on the ocellar nerve close to the retina became positive to one situated farther from the retina; on darkening, the polarity reversed. No nerve impulses were observed in the ocellar nerve; it was suggested that electrotonus in the nerve influenced the excitability of central elements, with anelectrotonic inhibition at "on" and catelectrotonic excitation at "off." Present study of ocellar responses in the grasshopper, *Oedaleonotus enigma* (Scudder), and the honeybee, *Apis mellifera,* does not confirm Parry.

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[1] This research was aided by a U. S. Public Health Service grant to Dr. T. L. Jahn.

[2] Present address: Pvt. Philip Ruck, Entomology Branch, Medical Laboratories, Army Chemical Center, Md.

Ocelli of *Oedaleonotus* or *Apis* (intact, head and thorax immobilized in plaster of Paris) were positioned almost in contact with, and looking directly into, the end of a stainless steel tube (no. 22 hypodermic needle) whose internal diameter was about equal to the diameter of an ocellar lens. The stimulating light (white) from a concentrated arc lamp (Buckingham and Dreibert, '46) was wholly confined within this tube. A fine stainless steel electrode, electrolytically pointed to 5–10 μ, and uninsulated, was passed through the exoskeleton at the edge of the lens and into the ocellar fundus or slightly beyond. The indifferent electrode was inserted under the cornea of a dark compound eye. DC amplification was used throughout. Negativity at an ocellar electrode gives an upward deflection in the figures. Stimuli were restricted to the ocelli; no electrical responses could be recorded from the compound eyes or other ocelli on illumination of the experimental ocellus. Rotating sector disks and Wratten neutral tint filters were used to control stimulus durations and light intensities. $\log I = 0$ is approximately 400 foot-candles. Experiments were run at 22°–23°C.

Six *Oedaleonotus* ocelli (from 6 animals), two median and 4 lateral, were studied. The on-effects of the lateral ocellus of figure 1 A consist chiefly of a sharp positive wave followed by a slower negative one; in some of the responses a slight negativity precedes the positive wave. The most interesting feature of the series occurs long after each stimulus has ended. In the first record a single "spike" appears with an off latency of almost 200 msec. With successive stimuli, additional "spikes" to the number of 4 or 5 appear; the off latency decreases progressively until, for the first ''spike'' of the last record, it is about 93 msec. After 50 seconds of such stimulation all the individual ''spikes'' had merged; the off latency of the summed complex was about 56 msec. The responses of the median ocellus in figure 1 B differ in that the negative slow wave is more prolonged, and in addition there is a positive wave at off. The long-delayed off complex is not fractionated as in A, and displays a variable amplitude. But in general the responses of A and B are similar. In all 6 ocelli, polarities of the slow response components were the same, and a long-delayed off complex was uniformly present. In one case the latter appeared in the early responses of a dark adapted ocellus, as in figure 1 A, but after the ''spikes'' had merged they disappeared altogether by the 30th stimulus and did not reappear until the ocellus was again dark adapted. In two of the ocelli, arhythmic background activity was observed in the unstimulated dark adapted ocellus, as recorded in the first trace of figure 1 B; the first stimulus removed this activity.

Of the 20 or so lateral honeybee ocelli which have been studied not one showed slow potentials of the graded sort commonly associated with electroretinograms or photoreceptor response. When the bee ocellar response is observed as a function of increasing light intensity its growth resembles a process of recruitment, synchronization and summation of units responding in a all-or-none manner, as figure 2 A illustrates. The flicker responses of figure 3 reinforce this impression. At a flicker rate of 11/sec. responses are very regular and in synchrony with each light flash. At rates of about 25/sec. synchrony is lost and wave forms become irregular. The upper 31.5/sec. record shows this. The lower 31.5/sec. record shows a partial, transient return to synchrony, but now at half the flicker rate. With careful adjustment of flicker rate, ''frequency halving'' can be regularly observed. Sixty per second approaches the flicker frequency at which ''spikes'' drop out, though there remains a residual in-phase undulation. The flicker fusion frequency (f.f.f.) for this undulation lies around 95/sec. when Log I = O. The undulation behaves like a graded potential insofar as it decreases gradually and regularly to the fusion point with increasing flicker rate.

The rather high f.f.f. of 95/sec. with a corneal illuminance of 400 foot-candles is indicative of rapid visual recovery processes in the bee ocellus. So is the dark adaptation experiment of figure 2 B; a test flash delivered 5 sec. after 5 minutes of exposure to Log I = O elicits a response, 4, of the same magnitude as the response, 1, to the same test flash when the ocellus was fully dark adapted. The f.f.f. value of 95/sec. compares with 160/sec. for the compound eye under exactly the same experimental conditions. If the f.f.f. of the ocellus increased with intensity at a rate proportional to that of the compound eye f.f.f., it would be roughly 150/sec. at 10,000 foot-candles, high enough to rank the ocellus among "fast" insect eyes (Autrum and Stoecker, '50; Autrum, '52).

Most of the data of these preliminary experiments merely raise questions, the answers to which will depend on refined technique. The origin of the responses cannot yet be stated with certainty. The uninsulated electrode generally passed through the receptor layer, and the tip lay in the post-retinal synaptic area. The records could therefore include an aggregate of sense cell slow potentials, nerve impulses in receptor cell fibers, and in the second order giant fibers (Parry, '47; Cajal, '18) of the ocellar nerve, and synaptic potentials. The off "spikes" of *Oedaleonotus* are probably post-synaptic in origin, and hence represent impulses in the giant fibers, an inference supported by the fact that the number of "spikes" does not exceed that of ocellar nerve fibers in *Locusta* (Parry, '47). The very long off latency in *Oedaleonotus* is interesting and not at all understood. Neither is it known why graded sensory potentials do not definitely appear in the bee ocellar response; perhaps they are present but masked by relatively much larger all-or-none nerve fiber activity.

## ACKNOWLEDGMENTS

The author thanks Dr. J. N. Belkin for supplying specimens of *Oedaleonotus enigma* and varifying the specific name, Dr. T. H. Bullock and Dr. T. L. Jahn for their friendly advice and critical reading of the manuscript.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Responses of *Oedaleonotus enigma* ocelli to stimuli of ⅛ sec. duration at Log I = O presented at a rate of 1/sec. Ocelli were dark adapted prior to the first stimuli. Signal marker on lower trace. A: lateral ocellus showing a discharge of discrete off ''spikes'' with long latency. B: median ocellus showing prominent slow potentials; a long latency off complex present but not fractionated as in A.

2 Responses of *Apis mellifera* lateral ocelli to stimuli of ⅛ sec. duration. A: effects of varying light intensity showing ''spikes'' which recruit and sum, with shortening latency and absence of slow waves. B: effects of light adaptation showing absence of amplitude change; 1, 2, and 3 are consecutive 1/sec. stimuli at Log I = O; ocellus was fully dark adapted before 1; 5 minutes of light adaptation at Log I = O followed 3; 5 sec. after light adaptation ended 4 was recorded also at Log I = O. Signal marker on lower trace.

3 Responses to flickering light of the lateral ocellus of *Apis mellifera*. Log I = O; light: dark ratio = 1; light on with downward deflection of signal marker (lower trace). ''Spikes'' drop out early, a diminishing flicker component fuses above 60/sec.

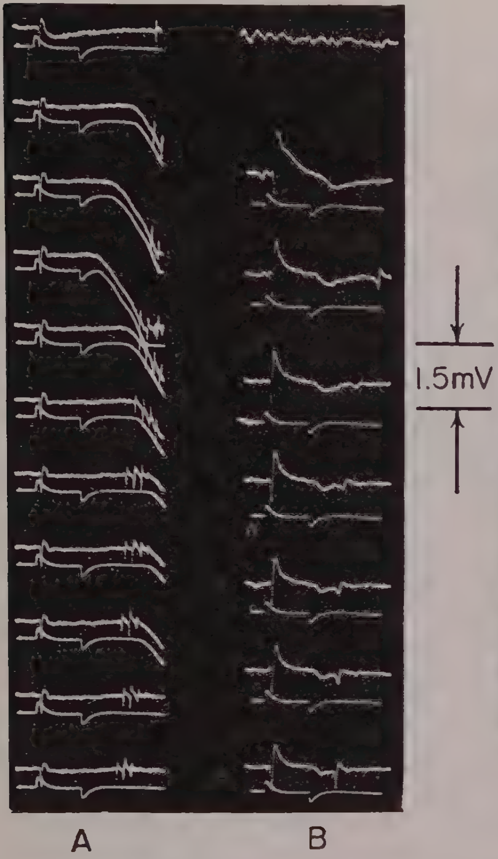


FIG. 1

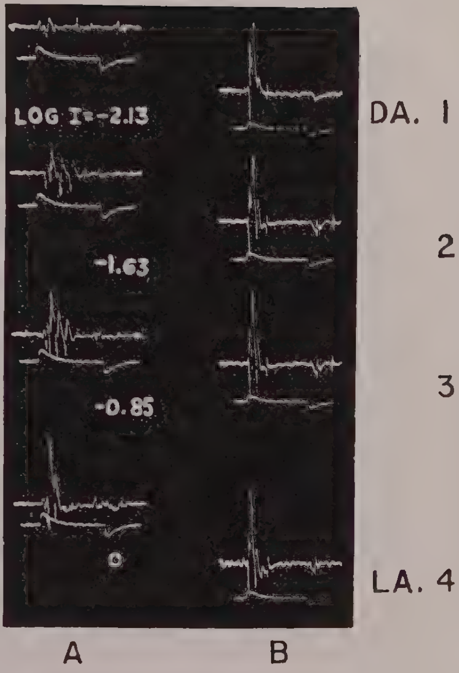
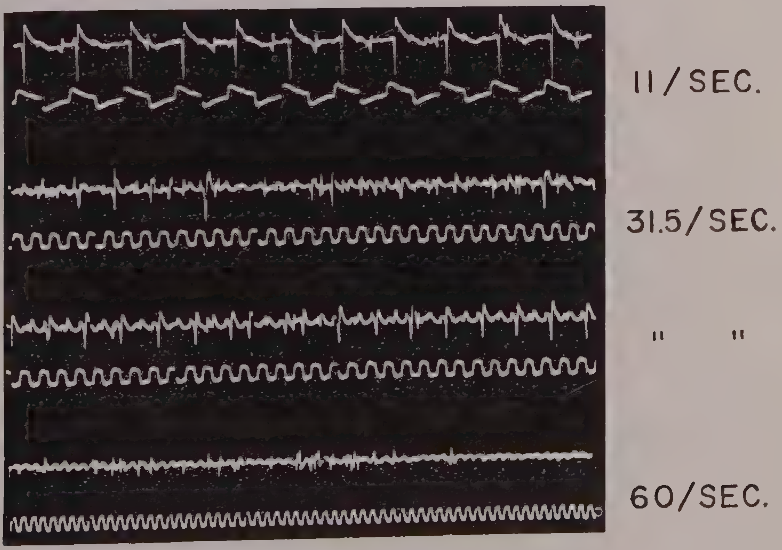


FIG. 2

FIG. 3



# EDITORIAL TRANSITION: RESIGNATION, APPRECIATION AND SUCCESSION

DETLEV W. BRONK
*Rockefeller Institute, New York 21, New York*

June of 1954 marked the end of 15 years during which I had served as Managing Editor of the Journal of Cellular and Comparative Physiology. The issue of that month completed the last of 30 volumes that had been published during the term of my service. In those years the number of subscribers had increased to more than double. It was an appropriate time for transfer of my editorial duties to another. Accordingly, I renewed my pleas that this be done as I had first requested two years before.

The duties that have been mine were made pleasant by the kindly and tolerant response of authors to my requests for revision, or to my reluctant rejection of their manuscripts. My greatest rewards from the 15 years of editorial labor have been the countless friendships that have developed in the course of correspondence concerning the thousands of papers that were submitted.

Four other debts of gratitude I would acknowledge. From E. Newton Harvey, who was the founder of the Journal and my predecessor, I inherited high standards of excellence. To my colleagues of the Editorial Board and to many others I gladly give warm thanks for loyal, selfless aid in formulating editorial judgments. Grace Lawton and James S. Ream of the Wistar Institute and their companions in the Publication and Press Department have labored faithfully and well to create the 90 issues from the manuscripts I delivered to them.

My associates, our friendly authors and subscribers, have enabled us to make the Journal of Cellular and Comparative

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Arthur K. Parpart of Princeton University has been selected as my successor. It is my pleasant privilege to report that he has generously agreed to assume the responsibilities I now relinquish. I bespeak for him the loyal support I have enjoyed.

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